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# Dynamic Excitations in Membranes Induced by Optical Tweezers

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## Keywords

Laser tweezers, Bilayer vesicles, Pore formation, Entropic forces, Colloidal forces, Hydro-dynamic instability

## Disciplines

Physical Sciences and Mathematics | Physics

# Dynamic Excitations in Membranes Induced by Optical Tweezers

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## Abstract

We present the phenomenology of transformations in lipid bilayers that are excited using laser tweezers. A variety of dynamic instabilities and shape transformations are observed, including the pearling instability, expulsion of vesicles and more exotic ones such as the formation of passages. Our physical picture of the laser–membrane interaction is based on the generation of tension in the bilayer and loss of surface area. While tension is the origin of the pearling instability, it does not suffice to explain expulsion of vesicles, where we observe opening of giant pores and creeping motion of bilayers. We present a quantitative theoretical framework to understand most of the observed phenomenology. The main hypothesis is that lipid gets pulled into the optical trap by the familiar dielectric effect, is disrupted, and finally gets repackaged into an optically unresolvable suspension of colloidal particles. This suspension can in turn produce osmotic pressure and depletion forces, driving the observed transformations.

*Condensed title:* Membrane Excitations

*Six keywords:* Laser tweezers; Bilayer vesicles; Pore formation; Entropic forces; Colloidal forces; Hydrodynamic instability.

## 1 Introduction

The function of biomembranes as tough flexible partitions of cellular organelles involves a rich diversity of dynamic phenomena. Processes involving membrane shape transformations such as exocytosis are controlled by the specific action of molecular machinery, which transduces readily available chemical energy and uses it to overcome viscous damping and elastic energy barriers (Alberts *et al.*, 1989). On the micron scale, cell membranes typically inhabit an environment out of equilibrium. Their motion is governed by the interplay between thermal energy, strong dissipation due to the surrounding fluid, and chemical energy. The thermal part is not only responsible for the incessant fluctuations of bilayers, but also leads to nontrivial entropic forces (Helfrich & Servuss, 1984; Evans & Rawicz, 1990). From a physical point of view, it is of interest to come up with simplified artificial membrane systems that can function as ‘micromachines’ in a

thermal environment. Can a system with no biological components exhibit dynamic processes similar to those occurring in the biological realm? We present here such a construction, composed only of lipid and water, with laser tweezers as the energy source.

Artificial membrane vesicles made of lipids (“liposomes”) have been used extensively as model systems to study the physical properties of lipid bilayers. These include elasticity, equilibrium shapes and shape transitions, fluctuations and adhesion (Deuling & Helfrich, 1976; Lipowsky & Sackmann, 1995; Lipowsky, 1991; Seifert, 1997). It is now widely recognized that most of the *equilibrium* properties of bilayers, on length scales much larger than the bilayer thickness, can be explained within the framework of the curvature elasticity model (Canham, 1970; Helfrich, 1973; Evans, 1974). This includes studies which have focused on relaxation dynamics in thermal equilibrium (Brochard & Lennon, 1975; Schneider *et al.*, 1984; Döbereiner *et al.*, 1995). Dynamic excitations of bilayers which take the membrane *out of equilibrium* have been less well studied, mainly due to the experimental difficulty in producing controlled perturbations in bilayers.

Our approach follows the spirit of the micropipette aspiration technique, which allows one to apply small forces on the membrane and measure the elastic response (Evans & Needham, 1987; Evans & Rawicz, 1990; Elbaum *et al.*, 1996). Laser tweezers have added a new tool to apply weak forces (Ashkin, 1970; Ashkin, 1980; Ashkin *et al.*, 1987; Svoboda & Block, 1994; Simmons *et al.*, 1996). To date the technique has mainly been used to manipulate micron size particles and to measure forces acting in macromolecular function (Ashkin, 1997). The novelty of applying laser tweezers to *lipid bilayers* lies not in the ability to drag objects, but rather in the variety of remarkable qualitative transformations which they create in membrane structures. In addition, laser tweezers offer excellent spatial and temporal resolution not available with other techniques.

When trapping macroscopic three dimensional objects such as microbeads, one is concerned with the force applied by the tweezers. In contrast, when tweezers are applied to a two-dimensional surface we are interested in the energy transmitted per unit *area*, or in other words in the *surface tension* created by the laser. Laser-induced tension is one of the main ingredients of the theoretical picture to be elaborated below. Its existence is immediately clear when we tweeze a unilamellar giant floppy vesicle at a point along its contour. Within seconds of tweezing the vesicle loses some of its area and becomes spherical with no visible fluctuations, a clear sign of tension (Bar-Ziv *et al.*, 1995a).

Laser-induced tension proved to be the key to understanding our first new instability, the “pearling”



transition (Bar-Ziv & Moses, 1994; Nelson *et al.* , 1995). Long cylindrical vesicles subject to laser tweezing at a point undergo a dramatic shape transformation into a modulated structure of a string of pearls completely delocalized from the tweezing point. The origin of this instability is a competition between the induced tension and the bending elasticity of the bilayer, as we have shown; we have quantified and characterized it, yielding a satisfactory agreement between experiment and theory (Goldstein *et al.* , 1996; Bar-Ziv *et al.* , 1997a).

However, when examining the response of complex vesicular structures where small vesicles are encapsulated within larger ones, it becomes apparent that tension cannot be the only ingredient in the physical picture of the laser–membrane interaction. When a parent vesicle has become round by laser tweezing it can then expel an encapsulated daughter vesicle, either spontaneously after the laser was shut off, or by continuous tweezing. We will argue below that two new concepts are required to explain the observations: osmotic flow (Moroz *et al.* , 1997) and a new “colloidal creeping” mechanism to be introduced below. Both of these mechanisms are rooted in the observation of irreversible loss of membrane under the laser action. We will propose a picture in which the area detached from the bilayer by the laser is repackaged in the form of a suspension of small particles (membrane fragments) which can produce osmotic effects and depletion forces similar to those observed in other colloidal systems.

Several of the phenomena described in this paper were announced in earlier publications (Bar-Ziv & Moses, 1994; Nelson *et al.* , 1995; Bar-Ziv *et al.* , 1995a; Bar-Ziv *et al.* , 1995b; Goldstein *et al.* , 1996; Moroz *et al.* , 1997; Bar-Ziv *et al.* , 1997a). The purpose of this paper is to bring these results together, to describe new results not previously described, and to create a unified physical picture capable of explaining most of them. The organization of the paper is as follows: in section II we present our physical picture for the interaction of a focused laser beam forming the optical tweezers with a fluid lipid bilayer. Section III is devoted to experimental procedures, protocol and data analysis methods. In section IV we review the pearling instability which is currently our best studied laser-induced dynamical shape transformation, while in section V we present new results on expulsion in closed vesicles. In section VI we present excitations of bilayers in planar structures where the laser induces local unbinding and can create topological excitations. Finally, we present new results on exotic excitations of vesicles with a high surface to volume ratio.

## 2 Physical picture

In this section we will sketch a unified physical picture of the interaction of laser tweezers with a lipid bilayer, then review the qualitative experimental evidence for the specific elements of this picture. In the sequel we will then use the picture to understand, in some cases quantitatively, several of the most striking membrane transformations.

### 2.1 Laser induced tension

The heart of the experiment is a  $\lambda = 514$  nm Argon laser and an optical microscope with a strongly focusing objective lens. Giant vesicles of various sizes and shapes are prepared by swelling pure lipids (typically DMPC) in water. The optical trap is produced by focusing up to  $I = 50$  mW of laser intensity from a beam 6 mm in diameter into a spot of size  $w_0 \approx 0.3\mu\text{m}$  (Svoboda & Block, 1994; Simmons *et al.*, 1996). A strong gradient of light intensity is set at the focus of the objective lens and a huge power density, 20 megawatt/cm<sup>2</sup>, passes through the D=4 nm thick bilayers under study. Most of this energy just passes through with minimal absorption and heating because the membrane is a thin transparent material at the laser wavelength (see below). However, a tiny amount of electromagnetic energy,  $U$ , does interact with the lipid and goes into polarizing it. This is

$$U = (\epsilon_L - \epsilon_W) \int \langle |E|^2 \rangle dV, \quad (1)$$

where  $\epsilon_L$  and  $\epsilon_W$  are the dielectric constants of the lipids and water in the visible range, and  $\langle |E|^2 \rangle$  is the time averaged electric field intensity. The integral extends over the volume of interaction of the electromagnetic field with the membrane. Since the thickness of the membrane  $D$  is much smaller than its lateral size we can transform the integral into a surface integral over the area of the membrane in the trap. Using  $\langle |E|^2 \rangle = I/w_0^2 c$  with  $c_W$  the speed of light in water we get:

$$U = \Sigma_L \int_{S_t} dS \quad (2)$$

where

$$\Sigma_L \equiv \frac{(\epsilon_L - \epsilon_W)ID}{c_W w_0^2} \approx 4.5 \cdot 10^{-5} \text{erg cm}^{-2} / \text{mW} \cdot I \quad (3)$$

Due to the strong focusing of the gaussian beam, the integral extends over an area of  $S_t \approx w_0^2$  which is much smaller than the total area of the vesicle.  $\Sigma_L$  is an *upper limit* on the tension which could be induced in the

membrane. In practice the actual tension will reflect only the *net* energy gain  $\sigma$  after the membrane has been folded to fit into the trap (see §2.4 below). For laser intensity  $I = 10$  mW,  $\Sigma_L \approx 5 \times 10^{-4}$  erg/cm<sup>2</sup>. As we will see, this is comparable to the tension needed to induce shape transformations in micron-size membrane structures. The corresponding interaction energy is  $U \approx 10^{-12}$  erg, comparable to the bending rigidity of the bilayer and not much more than the thermal energy  $k_B T$ . It is important to note that due to the diffraction limit,  $w_0 \propto \lambda$ . Therefore, the laser induced tension drops significantly with wavelength,

$$\Sigma_L \propto 1/\lambda^2, \quad (4)$$

favoring in this case the use of an Argon laser over near IR lasers such as the commonly used 1064 nm YAG laser.

## 2.2 Membrane elasticity and entropic tension

What is the response of a lipid membrane to a local tension of  $10^{-4}$ – $10^{-3}$  erg/cm<sup>2</sup>? To answer this question we must recall some elements of membrane elasticity (for a recent textbook see (Safran, 1994)). Lipid molecules self-assemble strongly into bilayer membranes. Any deformation of the membrane which changes the packing of molecules from their optimum arrangement will incur an elastic free-energy cost, but some deformations are far less costly than others. For example, *bending* a bilayer deforms its individual monolayers, but by a small amount proportional to  $D/\ell$  where  $D$  is the membrane thickness and  $\ell$  the radius of curvature. Indeed bends on the micron scale are so much easier than net stretching of the membrane that we may neglect the latter completely. This will be true so long as the applied tension  $\Sigma$  remains much less than the intrinsic stretch modulus of the membrane, which is the regime of interest to us. The free energy cost of a membrane configuration is then given by

$$E = \frac{\kappa}{2} \int \left( \frac{1}{R_1} + \frac{1}{R_2} \right)^2 dS, \quad (5)$$

where  $\kappa \approx 10 - 15 k_B T$  is the bending rigidity,  $R_1$  and  $R_2$  are the principal local radii of curvature and the integral is over the surface of the membrane, whose total area is fixed.

At zero temperature an ideal unconstrained membrane will adopt its preferred surface density of lipid molecules and thus attain zero tension. At finite temperature thermal fluctuations soften the membrane and effectively render it stretchy: a weak external force acting on the membrane can unfold thermal wrinkles, increasing its apparent area without actually changing the microscopic surface density of molecules in the

bilayer. Conversely, constraining the thermal fluctuations in a membrane lowers the entropy and generates a free energy cost per unit area, or in other words an effective tension. For example, fluctuations can be constrained by the combined constraints of fixed number of lipid molecules and fixed enclosed volume. Thus a closed vesicle will always have some nonzero surface tension. This has been established theoretically and beautifully demonstrated experimentally by Evans and coworkers (Helfrich & Servuss, 1984; Milner & Safran, 1987; Evans & Rawicz, 1990; Seifert, 1995a). For  $10\mu\text{m}$ -size lipid vesicles, under no applied force, tension values are as low as  $10^{-6}$  erg/cm<sup>2</sup>. In fact, this ‘entropic elasticity’ persists over 5 orders of magnitude, up to 0.1 erg/cm<sup>2</sup>, before the bilayer becomes a normal linearly elastic material (Evans & Rawicz, 1990). We dwell on this point because the optical tweezers (somewhat fortuitously) operate in the middle of this entropic regime

Thus optical tweezers turned out to be a suitable tool to excite dynamic shape transitions in bilayers, essentially because of the clear separation of energy scales between the soft, low energy, bending modes of the membranes and the highly energetic stretching modes. Laser induced tension of  $10^{-3}$  erg/cm<sup>2</sup> is a weak tension excitation compared with the stretching modulus  $\sim 100$  erg/cm<sup>2</sup>, but high when compared with the equilibrium tension  $\sim 10^{-6}$  erg/cm<sup>2</sup>.

### 2.3 Dynamics of tension and shape transformations

Since the membrane is a two-dimensional fluid, we should think of its surface tension as a negative 2d *pressure*. What we have argued, then, is that the effect of the laser is to maintain this pressure at some value  $\Sigma$  just outside the trap. What happens next depends crucially on the initial membrane configuration.

Tweezing an infinite planar membrane, or a very large flaccid vesicle, will simply result in a slow inflow of material to the trap. The flow will be impeded by the viscous loss of the water it entrains (plus a negligible loss from the thin bilayer itself), so that the tension in the membrane becomes negligible outside a radius a few times the trap size. Indeed, when tweezing in this configuration we do observe free Brownian fluctuation of the membrane away from the trap.

More interesting phenomena can happen in other configurations. As mentioned earlier, a spherical vesicle has minimized its projected area for the given enclosed volume, and is under tension. Applying additional tension cannot further decrease its surface area without releasing volume. In this case material flows into the

trap until the tension is equal to  $\Sigma$  everywhere. If the laser is turned off at this point, then this equilibrium will be maintained. We will see below how this tense state can store enough energy to drive membrane reorganization. Perhaps most interesting, however, is the case of an initially *cylindrical* vesicle of radius  $R_0$ . Like the sphere, a cylinder cannot lose any surface area at fixed volume without changing to some other shape. Plateau studied this problem in the previous century (Plateau, 1873), and noted that a small periodic perturbation in the cylindrical shape could reduce area at fixed volume if its wavelength exceeded  $2\pi R_0$ . Indeed we have not seen an initial disturbance of wavelength shorter than this.

Moreover, due to the volume constraint the area loss (and hence the energy gain due to the work done by the laser) is proportional to the *square* of the perturbation amplitude. Since the cylinder was initially a metastable equilibrium shape, any shape perturbation costs some elastic bending energy, again proportional to the square of the amplitude. (In contrast, a flaccid vesicle *reduces* its bending energy by becoming more spherical.) Thus we get a competition: no shape change occurs unless  $\Sigma$  exceeds a critical value  $\Sigma_{\text{pearl}} \sim \kappa/R_0^2$  (Bar-Ziv & Moses, 1994; Nelson *et al.* , 1995; Goldstein *et al.* , 1996). Before the tension reaches this value the cylinder is *effectively rigid*; tension must therefore spread out through the incompressible membrane (as in the spherical case) instead of being lost to viscous drag as the vesicle shape rearranges locally (as in the flaccid vesicle case).

Indeed this situation persists even after  $\Sigma$  rises past the pearling threshold (Goldstein *et al.* , 1996). Here again the key is the confined geometry of a thin cylinder. The fluid volume which must rearrange for a shape change scales linearly with the amplitude of the modulation, while the energy gain from the laser scales with the square of the amplitude. Thus initially the modulation grows slowly, at a rate fixed by the viscosity  $\eta$  of water. Dimensional analysis suggests that it moves with velocity  $v_f \sim \Sigma/\eta$ , and indeed we found that this is an overestimate (see below). Tension, on the other hand, spreads rapidly in a nearly incompressible membrane of nearly fixed shape.

Thus we find that tension initially outruns the advancing shape transformation. While constant-velocity shape propagation eventually catches up to the diffusive spread of tension, it turns out that the membrane elastic modulus is so large that this does not occur in the observable region of the initial pearling propagation (Goldstein *et al.* , 1996): we may thus take tension to be a constant equal to  $\Sigma$  throughout the pearling instability.

## 2.4 Detachment and escape of lipids

When a lipid molecule enters the laser spot, displacing water, the system gains electric energy via eqn. (1). We found that continuous tweezing results in an irreversible loss of membrane surface area: for example, vesicles made round and tense by the laser action remain that way indefinitely. Over 100 square microns of membrane can disappear in this way. Thus even though we cannot resolve optically the details of this process, we can nevertheless infer that lipids detach and escape from the membrane. Usually the remaining membrane does not become darker, nor do blobs of bulk lipid form, so some of the lost material must form membrane fragments of size well below our  $\approx 200$  nm resolution. Moreover, the trap volume of about  $3 \cdot 10^{-14} \text{ cm}^3$  is too small to contain all the lost lipid, up to  $100 \mu\text{m}^2 \times 4 \text{ nm}$ .

We gain further evidence that lipid is not simply folding up inside the laser trap when we note that packing extra material into the spot would require bending the membrane, incurring a curvature energy penalty. If we assume that the membrane bends with a wavelength characterized by the trap size, we get a lower bound for the packing cost per unit area:  $\Sigma_c \gtrsim \frac{4\kappa}{w_0^2} \approx 3 \cdot 10^{-3} \text{ erg/cm}^2$ . Only for  $\Sigma_L > \Sigma_c$  would the membrane gain net energy proportional to  $\Sigma \equiv \Sigma_L - \Sigma_c$  by entering the trap. Experimentally we observe a threshold for tension effects at laser intensities of  $I_c \sim 1\text{--}10 \text{ mW}$ , which as we shall show below corresponds to  $\Sigma_c \sim 0.5\text{--}5 \cdot 10^{-4} \text{ erg/cm}^2$ . Either our estimate for  $\Sigma_c$  is too high<sup>1</sup>, or else we must deduce that the huge energy flux through the bilayer creates additional effects which supply the energy needed to repackage the lipids.

We propose that the lipid gets pulled inside the trap and then pinched off the vesicle as little membrane fragments as shown in Figure 1. We do not have a microscopic model for this process; while we argue below against global heating, we cannot rule out a local radiation effect on the lipids inside the trap, a fact which might cause fragmentation and assist lipid loss from the membrane.

Once formed, small fragments can easily escape the trap, making room for more membrane to enter. To understand why large, organized lipid structures like membranes are pulled into the trap, while small fragments escape, we recall that many small objects have much more *entropy* than a few large ones. Put differently, every fragment has thermal energy  $3k_B T/2$  regardless of size, while its confining energy well due

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<sup>1</sup>One could suppose that self-adhesion effects inside the trap reduce  $\Sigma_c$  somewhat, but the required near-cancellation of the folding energy seems to us unlikely.

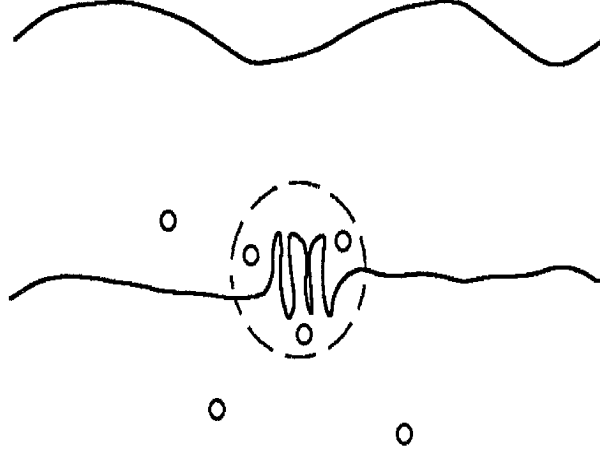


Figure 1: A cartoon of the effect of laser tweezers on a lipid bilayer. Lipid is packed into the laser spot by buckling and bending of the bilayer inside the laser spot. Eventually lipid fragments detach and escape the trap.

to the dielectric effect is proportional to its total number of lipid molecules. Thus while small fragments are disfavored due to their large curvature energy, only large fragments can remain trapped in the laser spot by the dielectric effect; the escaping fragments will be dominated by those smaller than

$$r_{\text{colloid}} \lesssim \left( \frac{3k_B T}{8\pi \Sigma_L} \right)^{1/2}.$$

For typical laser intensities we find that  $r_{\text{colloid}} \lesssim 20$  nm, indeed below our 200 nm resolution. *For illustration*, below we will make the perhaps optimistic estimate that the fragments are 5 nm in radius, a typical size for micelles, though the lipids in question do not spontaneously form micelles (Marsh, 1990). Vesicle suspensions nearly this small can readily be created by extrusion or sonication and are long-lived (Goll & Stock, 1977; Goll *et al.*, 1982).

We will argue in §5.2.3 that this irreversible loss of membrane into solution and the subsequent colloid of small particles thus formed in closed vesicles furnishes the driving force for laser induced spontaneous expulsion.

## 2.5 Colloidal effects

In the previous subsection we argued that the laser could create a colloidal suspension of small membrane fragments. Any such particles created outside a confined region will simply diffuse away, while those created inside a vesicle remain trapped and can lead to a number of surprising effects.

As long as the vesicle wall retains its integrity, any osmotic activity of membrane fragments will be negligible due to the far greater number of unavoidable small solute particles. The latter will clamp the vesicle volume due to their extremely slow diffusion through bilayer membranes (Alberts *et al.*, 1989). Thus for example we expect no colloidal effect in the case of pearling: here there is very little area loss, the tweezing times can be quite small (under a second), and there is no membrane gap to permit osmotic flow.

Suppose however that a hole opens in the membrane, of size intermediate between small solutes (like sugar) and  $r_{\text{colloid}}$ . Then small solutes will exchange freely, leaving only the osmotic effect of the membrane fragments. Since the large fragments are concentrated inside, the net effect is to *pull water into the vesicle*, and thus create an interior pressure. In (Moroz *et al.*, 1997) we argued that this effect supplies the driving force for vesicle expulsion.

Another well-known colloidal effect is the “depletion interaction” (Israelachvili, 1991). Normally one thinks of two rigid surfaces in a colloidal suspension. Each surface of area  $A$  is surrounded by a small “depletion volume”  $Ar_{\text{colloid}}$ : the center of each colloidal particle must stay outside this volume. The entropic cost of this forbidden volume can be eliminated by bringing the two surfaces into contact, eliminating depletion volume and leading to a free energy gain. Equivalently we can imagine the two surfaces being held together by an effective *suction* given by the osmotic formula,  $p_{\text{eff}} = -3k_{\text{B}}T\phi/4\pi(r_{\text{colloid}})^3$ , where  $\phi$  is the volume fraction. We get an upper bound on this pressure by taking  $r_{\text{colloid}} \sim 5$  nm, the size of micelles, and  $\phi \sim 0.3$ . Then  $-p_{\text{eff}} \lesssim 2 \times 10^4$  erg/cm<sup>3</sup>, a sizable pressure indeed.

We will see in §5.2.3 that inside a large vesicle the expected volume fraction is actually many thousands of times smaller than unity, since the laser destroys only a limited amount of membrane. However, in more confined spaces such as the thin layer between two bound bilayers,  $\phi$  can indeed approach unity, leading to large effects. Suppose for example that a bilayer edge is in contact with a second bilayer, as occurs during vesicle expulsion. The sliding contact keeps a  $\phi \sim 1$  suspension on one side separated from pure water on the other. In this situation the sliding edge will feel an entropic *force* pushing it towards the colloid side, since motion in this direction reduces the area exposed to the colloid and hence the entropically-costly depletion volume, with no corresponding increase on the pure-water side. We call this effect “colloidal creeping”.

The free-energy gain from colloidal creeping can also be considerable: multiplying  $p_{\text{eff}}$  by  $r_{\text{colloid}}$  in the above example gives an upper bound of 0.01 erg/cm<sup>2</sup>, again in the range of interest to us and, as we will see



in §5.3.2, sufficient to enlarge a membrane pore once it forms.

## 2.6 Experimental evidence for the physical picture

Before giving our detailed procedures and observations, we will give some qualitative facts and simple estimates supporting the general physical picture sketched in the previous subsections.

First we verify that the laser can apply forces of the expected magnitude. Figure 2 shows the result of pulling a very large flaccid vesicle. We show a section, about  $80\mu\text{m}$  long, of a much larger giant DGDG vesicle. Tweezing at a point (arrow in (b)-(d)) we were able to deform the membrane contour by slowly dragging it against the restoring elastic forces. Beyond a certain deformation (d) the restoring force overcame the optical force and the membrane popped out of the trap, then slowly relaxed back to the thermal equilibrium contour of (a). Throughout the process the vesicle continued to exhibit visible thermal fluctuations, indicating that the induced tension is negligible, as expected from our discussion in §2.3 above.

The maximal applied optical force can be estimated by measuring the deformed contour at mechanical equilibrium (d). The contour (d) subtracted from (a), was approximated by a best fit to a Lorentzian

$$h(x, y) = \frac{h_0}{(x^2 + y^2)/a^2 + 1}, \quad (6)$$

with  $h_0 = 16\mu\text{m}$  and  $a = 3.4\mu\text{m}$ . We computed the elastic bending energy  $E = \frac{1}{2}\kappa \int (\nabla^2 h)^2 dS$  with  $\kappa = 0.4 \times 10^{-12}$  erg the bending modulus and  $dS$  the surface element. Integrating over the surface deformation gives  $E = 3 \times 10^{-11}$  erg. The optical force balancing the elastic restoring force is roughly  $\frac{\partial E}{\partial h_0} \approx 4 \times 10^{-8}$  dyn. The tension associated with such a force is ultra-low,  $\Sigma \sim F/h_0 \approx 2 \times 10^{-5}$  dyn/cm, comparable to the minimal value for a flaccid vesicle mentioned in §2.2 above.

We can further verify the magnitude of the optical force deduced from the mechanical pulling experiment by dragging a spherical vesicle using the tweezers. This is a standard procedure to calibrate maximum trapping forces on spherical beads (Svoboda & Block, 1994; Simmons *et al.*, 1996). For a given trapping intensity there is a maximal dragging velocity  $V$  above which the trapped vesicle will pop out of the trap. At that velocity the optical trapping force and the Stokes' drag force coincide. Typically we measure  $F = 6\pi\eta RV \approx 10^{-8}$  dyn, or 0.1 pN. This result is at least an order of magnitude less than the trapping force measured on beads of similar size, due to the fact that the membrane is a two dimensional object and hence the optical force is weaker.

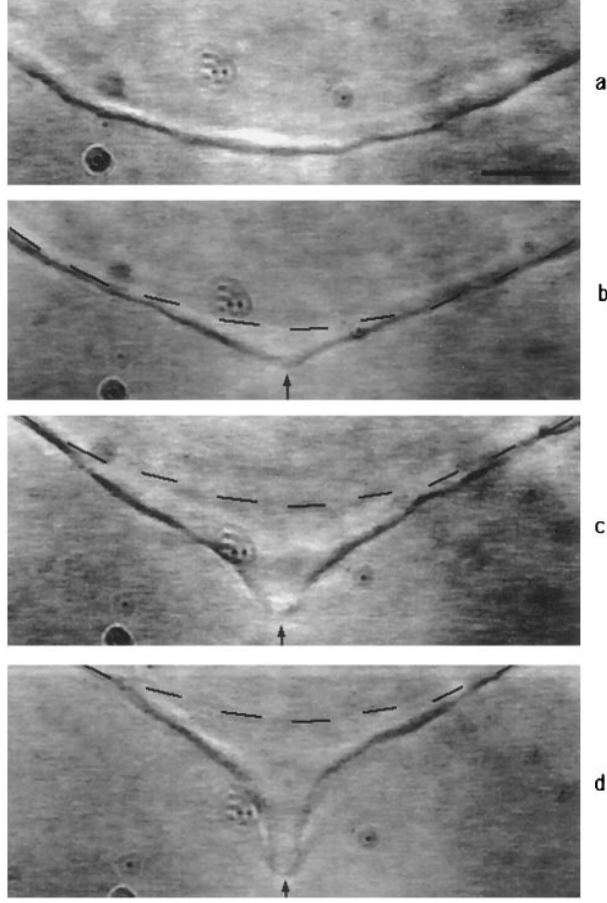


Figure 2: Mechanical manipulation of a membrane. The line is a two dimensional section from a DGDG giant vesicle that spanned the whole of the sample cell, and filled more than our video frame. The vesicle was observed after hours of swelling in the sample cell. The dashed line in (b)-(d) indicates the undeformed contour of (a), the arrow defines the point of tweezing and the bar is  $10\mu\text{m}$ .

Turning to the specific phenomena of interest to us, we mentioned in §2.3 that a cylindrical vesicle under sudden, uniform tension should have a *threshold*  $\Sigma_{\text{pearl}} \sim \kappa/R_0^2$  below which no shape transformation occurs. Indeed we have observed that for a membrane tube of radius  $R \approx 1\mu\text{m}$  made from DMPC lipid with a bending rigidity  $\kappa \approx 10^{-12}$  erg, there is a threshold laser power for pearling which corresponds under equation (3) to  $\Sigma_{\text{pearl}} \approx 10^{-4}$  erg/cm<sup>2</sup>, as expected, a direct confirmation for the energy scale of the laser-membrane interaction.

Tension alone cannot explain all our phenomena, however. In the expulsion experiment the laser puts a vesicle of radius  $R$  under tension, but then is shut off. The vesicle then spontaneously opens a pore to

allow the expulsion of an interior object of radius  $r$ . One might naively suppose that the surface tension pulls open the pore. But an  $R = 4.5\mu\text{m}$  vesicle under tension  $10^{-3}\text{ dyn/cm}$  has area only 1% greater than the same membrane under zero external tension (Seifert, 1995a). If the only energy storage mechanism were stretching of the membrane, all the stored energy would be spent by the time a pore of size 1% the total vesicle area had formed, or equivalently by the time a volume 1.5% the total vesicle volume had exited through this pore. Since the daughter object can have volume up to 50% the parent vesicle (see §5.2 below), some other mechanism must be forcing water into the space between the parent and daughter, pushing the daughter out. The only mechanism which can force water against a pressure gradient is *osmosis*, and yet initially the fluids inside and out were identical. In §5.2.3 we will argue that the colloid proposed in the previous subsection provides the required osmotic activity.

## 3 Experimental procedures

### 3.1 Setup

The optical tweezers were set up using an inverted microscope, a high numerical aperture lens and an Argon laser. The 488-514 nm Ar ion laser beam (Coherent, Innova 70) creates a tighter beam waist than infrared lasers and therefore a stronger electromagnetic field. Figure 3 is a schematic drawing of the setup. The inset is a close-up view of the vesicle suspension chamber with a temperature control system with excellent long term stability of 5 mK.

The suspension of vesicles was kept in a closed sample cell made from two thin cover slips sealed by wax or epoxy with a  $50\mu\text{m}$  mylar spacer. The cell was in contact with the objective lens on the bottom, while from the top it was attached with immersion oil to a sapphire window which was the bottom plate of a temperature controlled chamber. The temperature of the sapphire window and the objective lens was maintained using water flowing in a closed loop from a commercial heater-refrigerator bath (Lauda). The objective lens was also encased in a copper thermal sleeve encircling it, through which water from the temperature regulated bath flowed. In principle, the bath can stabilize temperature to within  $\pm 0.01\text{K}$ , but due to heat losses we achieved only  $\pm 0.1\text{K}$ . Fine regulation of the temperature within the vesicle cell was achieved by a thin foil heater (Minco) in the shape of an annulus placed between the sapphire and the vesicle cell. The temperature of the cell was monitored by a thermistor with negative temperature coefficient

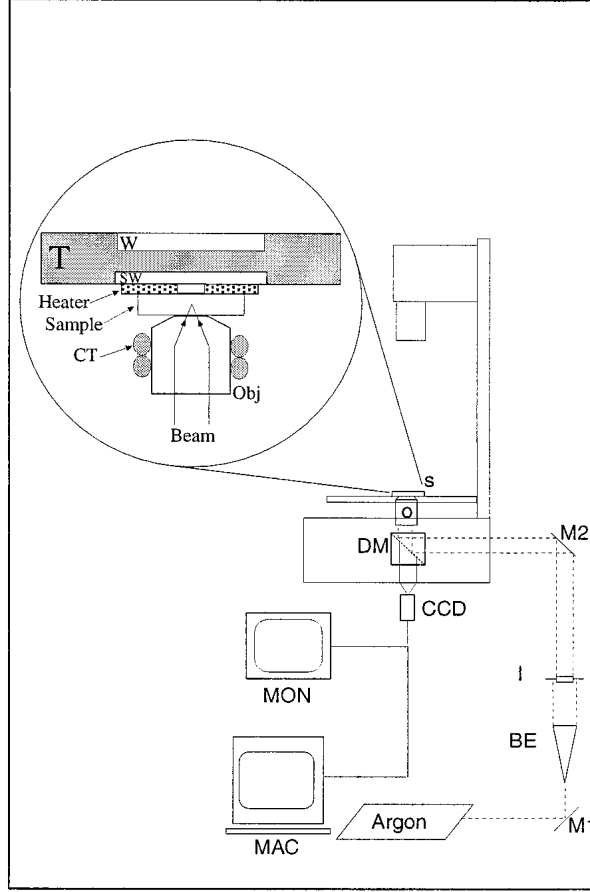


Figure 3: Argon: argon laser (Coherent Innova 70) operating in the 514.5 line. M1 and M2: mirrors. BE: beam expander composed of two positive lenses, I: iris diaphragm. Inverted microscope (Zeiss Axiovert 135). DM: dichroic mirror. CCD: CCD camera (Hamamatsu c2400 or Cohu 6500). MON: Monitor. O: microscope objective (Zeiss plan-apochromat Phase x63/1.4). Images are taken into the computer (Macintosh, Quadra 800) using a frame grabber (Data Translation) and Image software (NIH), S: sample. Inset: Obj: Objective Lens. CT: Copper tubing. Heater: thin foil 50 Ohms (Minco). SW: Sapphire window. W: optical window. T: temperature regulated stage.

having sensitivity of  $\sim 3000 \Omega/\text{K}$ , which constituted one leg of a Wheatstone bridge balanced by a precision (0.01%) resistance decade. The signal was fed back, through a differential integrator and amplifier<sup>2</sup> to a voltage controlled power amplifier heating the foil. The temperature was set such that the heater always heated in steady state. Using an integration time of  $\sim 10$  sec the system averages out fluctuations and obtains long term stability of 5 mK with about 1 Watt heating the foil.

<sup>2</sup>We are grateful to Y. Barad and V. Steinberg for providing us with their home made low noise instrumentation amplifier.

## 3.2 Materials and preparation

Vesicles were prepared from commercially available lipids (Sigma), dimyristoyl-phosphatidylcholine (DMPC), stearoyl-oleoyl-phosphatidylcholine (SOPC) and digalactosyl-diglyceride (DGDG), all of them uncharged, zwitterionic lipids, by using standard protocols (Evans & Needham, 1987) as described below. As far as we could tell our results were not specific to one type of lipid, and all the lipids we have used consistently displayed the same response to tweezing. In the following we shall not emphasize the chemical differences due to the overall similarity in behavior. We preferred to work with lipids whose melting temperature was conveniently below our ambient working room temperature. Lipid powder was added to a mixture of methanol and chloroform (2:1) at a concentration of 10 mg/mL. To produce giant vesicles we deposited about 20  $\mu$ L of lipid solution on a Teflon disk placed inside a beaker. The Teflon was thoroughly cleaned prior to being used and was roughened with emery paper, as described in (Evans & Needham, 1987). The methanol/chloroform solvent was allowed to evaporate and then dried under vacuum. This assists in obtaining a higher yield of unilamellar vesicles. Then a few mL of pure water or 0.1–0.5 molar sugar solution, heated to 40 °C, were added and the beaker was left in the oven over night at a temperature of 40 °C. Vesicle suspensions were readily identified as typical ‘clouds’ and were gently aspirated into a syringe, then transferred into the microscope cell. Glass components were cleaned by sonication in detergent and water, followed by multiple thorough rinses in ultra-pure water (Barnstead E-pure). This was followed by sonication in an organic solvent, usually ethanol, for about 10 minutes. The experiments were performed in the fluid state of the membranes in a closed cell, typically at 15 – 20°C above the fluid-gel transition point.

A different preparation technique incorporating a simple kinetic procedure was used to produce long cylindrical structures. A few microliters of lipid solution were deposited on a clean cover slip and left for a few hours under vacuum in a desiccator, to allow the solvent to evaporate. Two slices of mylar, 50  $\mu$ m thick, were placed on the glass as spacers and an upper cover slip was fixed to the bottom one using epoxy or melted wax, leaving two opposite sides unglued. Warm water was then injected from the side into the cell with a syringe. This induced a flow capable of carrying along globules of lipids, which started to swell from the deposit on the bottom cover slip. This injection procedure could be repeated a few times to create a number of strong flow pulses in the cell. Within a few hours of swelling we observed tubes oriented in the direction of the flow extending over hundreds of microns, typically anchored at both ends to lipid globules

which were still attached to the bottom cover slip. These structures remained metastable for hours to days.

### 3.3 Image analysis

Quantitative image analysis was carried out in order to follow dynamic shape transformations of vesicles. We implemented a fast digitization algorithm of the two dimensional contour extracted from the frame grabber, written as a user-defined interface within the NIH Image software. Döbereiner has presented excellent image analysis procedures to study vesicle shape transformations (Döbereiner, 1995). A contour of a membrane obtained with phase contrast microscopy is approximated by the contour perpendicular to the steepest gradient of intensity along the typical halo profile. By measuring the intensity distribution of the video pixels one can locate the nominal line, to better than one pixel resolution, using a smooth interpolating scheme (Döbereiner, 1995). This kind of analysis is somewhat time consuming but is necessary for small shape changes.

In all of our shape changes we did not need such an elaborate algorithm and hence developed a faster algorithm which finds the membrane contour to within pixel resolution, compromising for some digitization noise. The algorithm probes the vicinity of a contour point by averaging the pixel intensity over 13 pixels in 8 directions and locating the contour point in the direction of maximum intensity, similar to (Duwe *et al.*, 1990). Once a set of points  $\{x_i, y_i\}$  is found we smooth out the contour and can proceed to analyze the shape changes according to need, as described in the specific sections below.

Once the contour was determined we could measure a number of quantities to follow the shape changes and the fluctuations. A simple way to follow the decrease of fluctuations during tweezing (§5.2.1) and adhesion deformation (§5.2.2) of spherical vesicles was to measure the fluctuations in the normalized mean square of the radius. These are defined as

$$\frac{\langle R^2 \rangle - \langle R \rangle^2}{\langle R \rangle^2}, \quad (7)$$

where,

$$\langle R \rangle = (1/N) \sum_i^N \left| \vec{R}_i(x, y) - \vec{R}_{CM} \right| \quad (8)$$

is the average radius of the contour relative to the center of mass, and similarly for  $\langle R^2 \rangle$ .

To quantify the shape fluctuations of open contours (§5.3.2) we transform to the arclength parameteri-

zation:

$$s_i = s_{i-1} + \sqrt{(x_i - x_{i-1})^2 + (y_i - y_{i-1})^2} \quad (9)$$

$$\Psi_i = -\arctan\left(\frac{y_{i+1} - y_{i-1}}{x_{i+1} - x_{i-1}}\right), \quad (10)$$

with proper care taken at the boundaries. From the starting point,  $s = 0$ , to the end point,  $s = s^*$ , we computed the tangent angle to the contour,  $\Psi(s)$ . For each contour we numerically computed two parameters: the “curvature integral”:

$$\left\langle \left( \frac{d\Psi}{ds} \right)^2 \right\rangle \equiv \frac{1}{s^*} \int \left( \frac{d\Psi}{ds} \right)^2 ds. \quad (11)$$

and the “surface integral”:

$$\langle \Psi^2 \rangle \equiv \frac{1}{s^*} \int \Psi^2 ds. \quad (12)$$

These two quantities will help us evaluate and distinguish the geometric contributions to the energy of the membrane, arising either from changing the surface or from changes in the curvature.

Finally, to analyze the shape deformations in detail for *axisymmetric* shapes (§5.3.3), we used the mode expansion method developed by Döbereiner (Döbereiner, 1995). For each frame we rotated and translated the contour so that it was aligned with the major axis of the inertia tensor. One can then parameterize the vesicle contour by the tangent angle to the contour arclength  $\Psi(s)$  measured for each half contour from the north pole,  $s = 0$ , to the south pole at  $s = s^*$ . Thus,

$$\Psi(s) = \pi \frac{s}{s^*} + A_1 \sin\left(\pi \frac{s}{s^*}\right) + A_2 \sin\left(2\pi \frac{s}{s^*}\right) + A_3 \sin\left(3\pi \frac{s}{s^*}\right) + \dots \quad (13)$$

$A_2$  measures the ellipsoidal deformation of the sphere (Döbereiner, 1995)

$$A_2 = 1 + \sum_i \left( \Psi_i \sin\left(2\pi \frac{s_i}{s^*}\right) + \Psi_{i+1} \sin\left(2\pi \frac{s_{i+1}}{s^*}\right) \right) \frac{s_{i+1} - s_i}{s^*}. \quad (14)$$

### 3.4 Heating and absorption

Working with focused laser and biological materials one is always concerned with radiation heating effects. The most direct evidence that there is no *global* heating of the water is that the membrane does not respond unless the laser is applied directly onto it. To further confirm this we measured the absorption directly, and estimated the amount of heating due to absorption at the laser wavelength by assuming that the trap is a

localized heat source (Block, 1990). The steady state temperature rise of the surrounding liquid is  $\Delta T = \frac{I_{abs}}{\lambda R}$  (Block, 1990) where  $I_{abs}$  is the intensity absorbed at the laser wavelength,  $\lambda$  is the thermal conductivity of the surrounding liquid and  $R$  is the distance from the heat source. The absorption inside a trap of size  $w_0$  is  $I_{abs} = I_0(1 - e^{-w_0/\xi})$  where  $I_0$  is the incident intensity and  $\xi$  is the absorption length of the material at the laser wavelength. We measured the absorption of a highly concentrated lipid solution (100 mg/mL DMPC in methanol-chloroform (1:1)). We found an *upper bound* of 0.024 on the optical density (absorbance), at 488 – 514 nm, from which we obtained an upper bound of 0.16 liter/mole-cm for the molar extinction coefficient,  $\bar{\epsilon}$  of lipids. We consider two extreme cases where (1) the membrane bilayer is entirely crumpled inside the trap at a concentration of  $\bar{c} \approx 0.45$  mole/liter and (2) the membrane is flat inside the trap at a concentration of  $\bar{c} \approx 6 \times 10^{-3}$  mole/liter. For the first case the absorbance inside the trap of size  $w_0 \approx 0.3\mu\text{m}$  is  $w_0/\xi \equiv \bar{\epsilon}\bar{c}w_0 \approx 2 \times 10^{-6}$  and for the latter case  $w_0/\xi \approx 3 \times 10^{-8}$ . Hence for an input intensity of  $I_0 = 50$  mW (at the trap) the upper limit of the *local* temperature rise is  $\Delta T \approx \frac{I_0}{\lambda w_0} \frac{w_0}{\xi} \approx 0.5$  K and  $5 \times 10^{-3}$  K, respectively. Both results for  $\Delta T$  should be taken as overestimates and upper bounds for the actual heating. For example we have neglected local cooling due to convection.

## 4 Pearling of cylindrical vesicles: a dynamical instability

‘Pearling’ is the peristaltic transition that a membrane tube which was stabilized by curvature elasticity undergoes when a sudden tension is applied to it. Of the many excitations and transitions that we have observed in membranes, this is the best characterized by far and has played an important role in our understanding of nonlinear dynamics in membrane structures. For one, it is experimentally well controlled. Second, it can be formulated as a theoretically tractable one dimensional problem. Third, it shows clearly the generation of tension in the membrane by the laser, a tension that interacts and competes with the curvature elastic energy, in a manner that lies at the heart of the action of the laser on membrane structures in general. Finally, it typifies many of the problems and aspects that arise with dynamical problems: Strong coupling to the surrounding flow, universal behavior and critical exponents at the transition, dynamical selection of velocities and wavelength, strong fluctuations and their interplay with the linear growth regime, and a complex evolution of the fully developed nonlinear regime. Clearly this is a rich system, exhibiting a variety of complex phenomena, yet it is simple enough to be understood in some depth.



Beyond establishing the existence of the instability, our efforts have mainly concentrated on elucidation of the onset and linear stage. Already this proved nontrivial, since the thermal fluctuations compete with the hydrodynamic modes at onset and mask the linear stage, which we were unable to stabilize. An experimental challenge would now be to slow down or otherwise control the linear stage, to measure the linear coefficients directly. Since we were unable to do that, we measured them indirectly, through the existence of propagating front solutions. While attempts to characterize the nonlinear stage (Bar-Ziv & Moses, 1994; Goveas *et al.*, 1997) look promising, this is clearly a different level of difficulty.

Since we reported the instability, numerous observations have been made on the excitation of pearls in a variety of other situations, usually associated with mechanically induced tension. Specially interesting examples are the pushing of the membrane from within by a microtubule (D. Kuchnir Fygenson, private communication) and the destabilization of a tether by pulling on a bead attached to the membrane (B. Pouligny, private communication).

Schematically, the mechanisms and stages in the progression of the pearling instability can be described by the following flow chart:

Laser action  $\rightarrow$  Competition of tension and curvature  $\rightarrow$  Instability  $\rightarrow$  Flow and Dynamics  $\rightarrow$  Threshold and onset  $\rightarrow$  Propagation  $\rightarrow$  Marginal Stability Criterion  $\rightarrow$  Quantitative comparison to Linear Theory  $\rightarrow$  Nonlinear regime.

## 4.1 Phenomenology

Our picture of the instability is as follows. The laser grabs the membrane at one point, and begins pulling in lipid from the membrane that is outside of the trap. This causes a loss of area in the rest of the membrane, and an effective tension in it. The instability begins at the point of tweezing and propagates outwards, at a rate that depends on the strength of tweezing, or applied tension. Figure 4 shows an example of a propagating, fully developed pearling instability. The linear sinusoidal perturbation rapidly increases in amplitude and coarsens. This tendency continues into the highly nonlinear stage, where the amplitude has peaked — making the pearls big and almost spherical while the diameter of the tube that connects between the pearls becomes extremely small.

Interestingly, if the pearling has developed into the nonlinear stage, then *after* the laser is shut off we

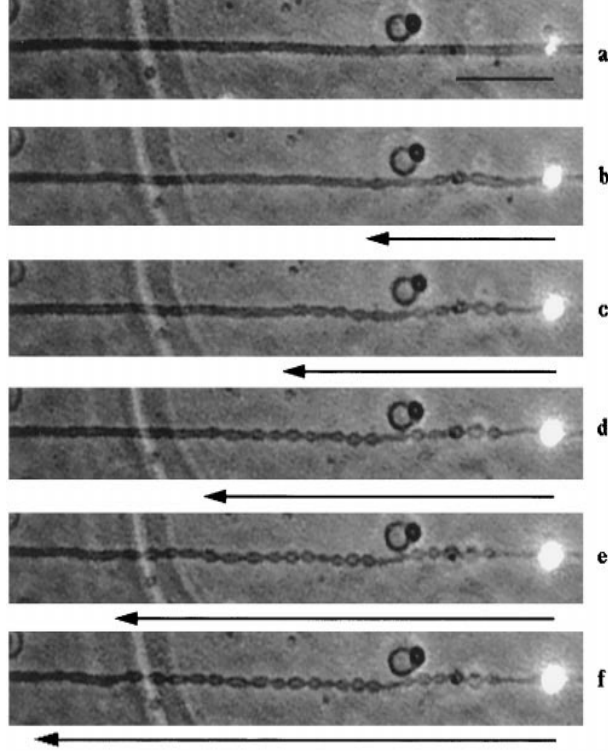


Figure 4: Propagation of a fully developed, large amplitude pearling front outward from the illuminated laser spot. Time between frames in seconds: (a) 0, (b) 0.44, (c) 0.6 (d) 0.84, (e) 1.08, (f) 1.14. The bar represents  $10\mu\text{m}$ .

observe that pearls flow back and migrate towards the point of tweezing, where they collect until the tension relaxes. Measuring a decay of migration velocity shows that there is a monotonic decrease of the tension after the laser is turned off.

A striking aspect of the instability, which differentiates it from other phenomena in membranes, is the nonlocal effect of the localized laser action. Nowhere else does a force, caused by the focusing of light down to a  $0.3\mu\text{m}$  spot, cause the instability of a membrane structure for hundred of microns away. This proves the global nature of the pearling instability, and dispels any worries about heating effects of the laser. This global instability is instead related to the classic Rayleigh instability of a cylinder under surface tension. An extreme example of this is given in Figure 5(a), where tubes that formed junctions with each other are seen to destabilize along all the different arms, and the pearling ‘jumps’ across intersections (b). Further evidence for the long range of the tension effect is seen in figure 6, where a collage is made of many adjacent video

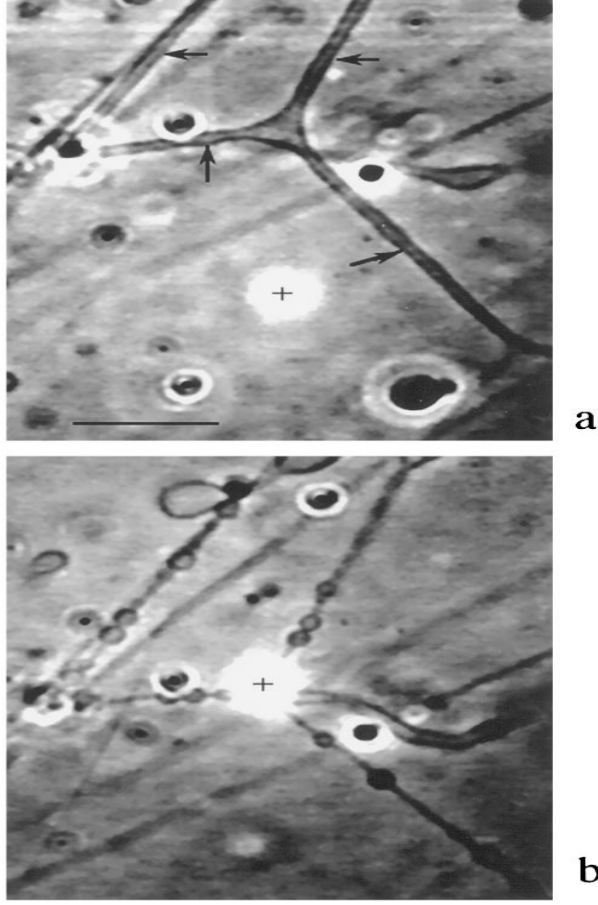


Figure 5: (a) A network of connected intersecting tubes made from DGDG lipids. The marks indicate different sections of the tube, and the crosses indicate tubes position of the laser tweezers. (b) Upon tweezing the tube at one place the instability propagates to all sections. The tube here has reached the nonlinear stage, and the pearls are stable over a long time. The bar represents  $10\mu m$ .

frames that cover a length of tube much larger than we usually viewed.

It is possible to induce pearling by mechanical tension instead of the laser induced tension, a fact which further strengthens our picture of the laser action. This was done by simple micromanipulation. We inserted a glass microneedle into the sample and pulled on a lipid globule which had tubes attached to it. A rapid enough pull caused elongation of the tubes and produced uniform pearling modes with both linear, small amplitude modulations and nonlinear, isolated pearl structures. This is shown in figure 7, where the glass microneedle (not shown) was pulling at the end of the tube. The instability initiated with uniform, small amplitude long modes and then transformed into isolated spherical pearls connected by thin tubes, exactly

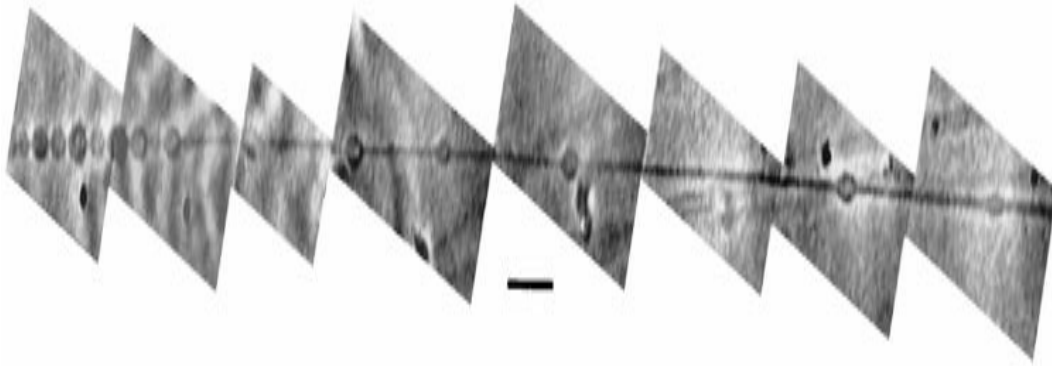


Figure 6: A collage of video frames showing the long range of the instability. The tweezing point was just to the left of picture. Bar scale is  $10\ \mu\text{m}$ .

as in the laser induced case. An additional example is presented in Figure 8, where we induced pearling by using the tweezers to drag a bead that was encapsulated in the vesicle wall.

Since a globule is an infinite reservoir of lipids one can, in principle, mechanically draw tubes of increasing length without inducing tension (and hence pearling) by pulling slowly so that new lipid can flow along with the pull from the lipid reservoir. However, if the pull is rapid and the tube long enough, lipid cannot flow from the globule and the surface area of the tube is effectively fixed. For the tweezers, no lipid is added near the trap. Thus membrane can only be delivered by a transformation to a new shape of equal volume but less area per unit length.

The production of tubes and their subsequent robustness is in itself an interesting issue. Our tubes are all metastable, ending up after many hours (about a day) as a large sphere on a very thin tube. The fact that these tubes come in a range of radii and lengths, and that they still evolve as we observe them, complicates the picture sufficiently that at present there is no explanation of the exact details of their production and evolution.

## 4.2 Overview of theoretical approach

### 4.2.1 Basic considerations and assumptions

The origin of the pearling instability of a membrane cylinder can be understood as a competition between the curvature elasticity which stabilizes the tube and an externally applied surface tension which destabilizes the tube (Bar-Ziv & Moses, 1994). As mentioned in §2.3, the destabilizing effect of tension arises from

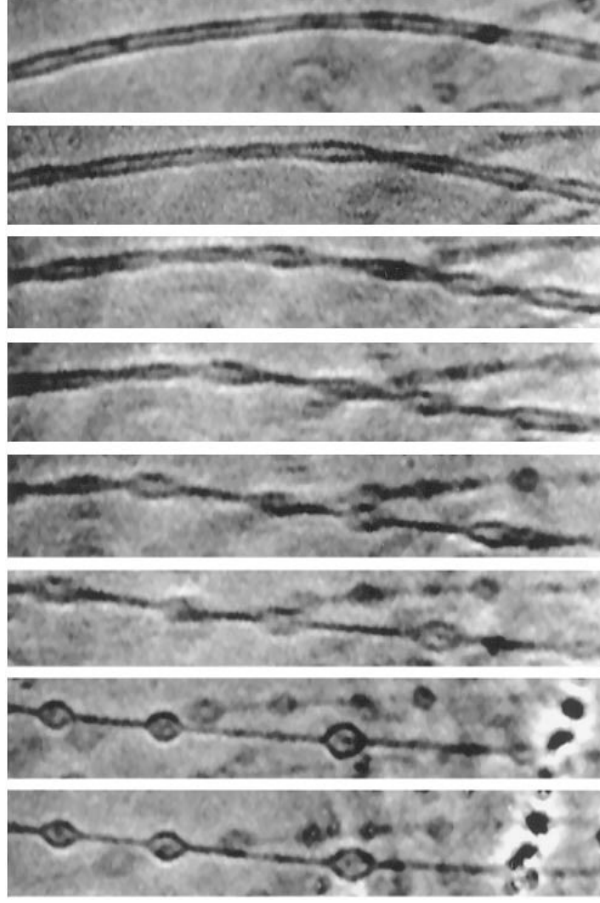


Figure 7: Pearling by a rapid mechanical pull using a glass microneedle. The needle (not shown, on right side of frame) was positioned perpendicular to the tube axis and the direction of the pull was parallel to the axis.

the geometrical fact that long wavelength deformations on a cylinder can reduce the surface area while maintaining its enclosed volume, thus satisfying the tendency of a positive surface tension to minimize area. The most simplified approach is to assume that the tension is constant in time and uniform along the tube. In §2.3 we recalled the argument of (Goldstein *et al.* , 1996) that the laser applies a sudden tension which spreads rapidly along the tube due to the incompressibility of the membrane. Thus tension exists in the membrane before any shape transformation can occur and one can analyze the initiation of the instability assuming a constant tension. The same argument holds for the mechanically induced instability where by applying a rapid pull on a globule with an attached tube we induce a sudden force per unit length (tension) along the tube before a change of shape can occur.

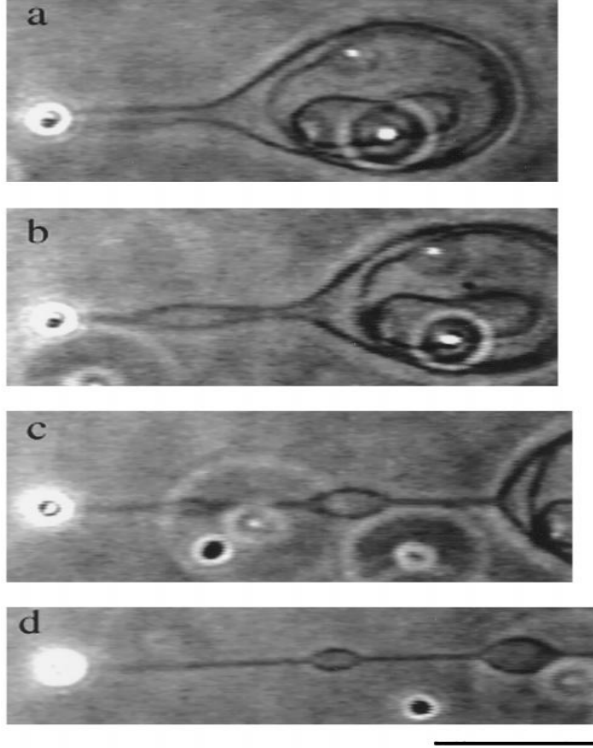


Figure 8: Pearling by several rapid mechanical pulls performed consecutively on a silica bead that was encapsulated in a vesicle and trapped by the tweezers. Typical time scale is seconds, and the tugging velocity was typically  $15\mu\text{m}/\text{sec}$ . Bar is  $10\mu\text{m}$ .

Granek and Olami have proposed an alternative time dependence of the laser-induced tension resulting from two competing effects: an assumed constant suction rate of surface area, rather than the constant *tension* assumed here, and the stabilizing tendency of the membrane to revert to zero tension (Granek & Olami, 1995). Their analysis predicted a coupling between the time dependent surface tension and the growth of the instability. We have not been able to address the time dependence of the tension in our experiment and have therefore assumed constant and uniform tension, at least in the initial growth of the instability.

Olmsted and MacKintosh have argued that gradients of tension are created in the laser experiment since at the far ends of the cylinder the membrane is attached to a lipid reservoir at zero tension (Olmsted & MacKintosh, 1997). However, our field of view in the microscope limits our observation to a few wavelengths on each side of the tweezing site, which is typically chosen to be quite far from the ends of the tube, and

hence we are insensitive to gradients.

#### 4.2.2 Linear stability analysis

Consider a membrane tube subject to uniform and constant tension. The energy of the membrane is then a sum of the curvature elasticity and a surface energy,

$$F = \kappa \int 2H^2 dS + \Sigma \int dS . \quad (15)$$

To identify the instability we must calculate the energy of small deformations of the shape and find those modes which reduce the energy of the unperturbed tube (Bar-Ziv & Moses, 1994; Nelson *et al.*, 1995; Granek & Olami, 1995; Gurin *et al.*, 1996). We limit ourselves to shape deformations that are axisymmetric and in which the axis is unperturbed (peristaltic modes). For a surface given in terms of the local radius,  $R(z)$ , in cylindrical coordinates the area element is  $dS = 2\pi R(z) \sqrt{1 + R_z(z)^2} dz$ , where the subscript denotes partial derivative, and the mean curvature  $H$  is:

$$H = \frac{RR_{zz} - 1 - R_z^2}{2R(1 + R_z^2)^{3/2}} . \quad (16)$$

Linear stability analysis permits us to restrict attention to sinusoidal perturbations of the form

$$R(z) = \rho_0 + u_q \sin(qz). \quad (17)$$

The unperturbed tube radius,  $R_0$ , is related to the amplitude  $\rho_0$  via the volume conservation constraint:  $\rho_0 = R_0 \sqrt{1 - u_q^2/2R_0^2}$ . Integrating and keeping terms to quadratic order in  $u_q$  we obtain the excess energy per unit length over the unperturbed tube,

$$f = \frac{1}{2} \frac{\pi \kappa}{R_0^3} \sum_k \left( \left( \frac{3}{2} - \sigma \right) + (\sigma - 1)k^2 + k^4 \right) u_k^2 \quad (18)$$

where the nondimensional wavenumber is  $k = qR_0$  and  $\sigma \equiv \Sigma R_0^2/\kappa$  is the normalized ratio of surface tension to curvature. For  $\Sigma = 0$  all wavenumbers are stabilized by the curvature. For  $\kappa = 0$  we have the Rayleigh instability of a cylinder of fluid, where all modes with  $k < 1$  are unstable as they reduce the surface area at constant volume (Rayleigh, 1892). For finite  $\Sigma$  and  $\kappa$  long wavelength modes become unstable only above a critical value,

$$\Sigma_{\text{pearl}} = \frac{3\kappa}{2R_0^2} . \quad (19)$$

### 4.2.3 Dispersion relation

While the linear stability analysis identifies the instability control parameter and the unstable modes it seems to predict that, for any  $\Sigma$ , the most unstable mode is  $k = 0$ , since that is where eqn. (18) is as negative as possible. Indeed, Rayleigh's original analysis of the instability of a viscous thread gave this conclusion, leading to an irregular breakup of the thread (Rayleigh, 1892). But the pearling instability selects a well-defined, nonzero wavenumber (Fig. (4)). To understand this discrepancy, note that eqn. (18) says nothing about the *rate* of growth of a mode. In fact modes with  $k \approx 0$  involve large scale fluid motion, a process which incurs strong hydrodynamic dissipation. To obtain the growth rates of the unstable modes and the fastest growing mode one has to solve the hydrodynamic equations of the fluid motion which balance the tension against the viscous dissipation under the appropriate boundary conditions.

We can easily carry out these steps using a rough approximation to the hydrodynamics. Neglecting the bending stiffness for the moment, eqn. (18) reduces to an energy for mode  $k$  whose time derivative is  $P_{\text{laser}} = \frac{\pi\Sigma}{R_0}(-1 + k^2)u_k\dot{u}_k$ . This is minus the rate at which energy is given to the system by the laser. To see where this energy goes, we note that in time  $\delta t$  a volume  $\delta V \sim (2\pi R_0/2k) \cdot (2\pi R_0) \cdot (\delta u/2)$  of fluid needs to be moved from the narrowing parts of the tube to the thickening parts. To do this while maintaining no-slip boundary conditions at the wall of the tube requires a central fluid velocity of  $v(0) = 2\pi\dot{u}/k$ , by the Poiseuille formula. The squared shear rate of this flow, times the viscosity  $\eta$  of water, is the rate of viscous energy loss per unit volume. Equating the total loss  $\eta\frac{16\pi^3}{5k^2}\dot{u}^2 R_0$  to  $-P_{\text{laser}}$  then gives the approximate growth rate:  $\omega(k) \equiv \frac{\dot{u}}{u} = \frac{5\Sigma}{16\pi^2 R_0^2}k^2(1 - k^2)$ . Thus we see that the growth rate indeed is not maximum at  $k = 0$ , but rather at  $k = 1/\sqrt{2} \approx 0.7$ , roughly as observed.

While the above derivation is transparent, quantitative comparison to the experiment requires a more accurate treatment (Goldstein *et al.*, 1996; Bar-Ziv *et al.*, 1997a). In particular, by neglecting bending stiffness we have missed the *threshold* phenomenon mentioned in the previous subsection. A useful approximate form for the complete dispersion relation is (Bar-Ziv *et al.*, 1997a)

$$\omega(k) = \tau_\kappa^{-1} b k^2 \left[ \frac{3}{2} \left( \frac{\Sigma - \Sigma_{\text{pearl}}}{\Sigma_{\text{pearl}}} \right) (1 - k^2) - k^2 - k^4 \right], \quad (20)$$

where  $b \approx 0.04$  and  $\tau_\kappa = \eta R_0^3/\kappa$ . The  $k^2$  multiplier is familiar from the simplified treatment in the preceding paragraph; it reflects the dissipation of long modes. The terms in the brackets originate from the curvature



and surface energies of the mode. The dispersion relation predicts a continuous transition with a selected wavenumber of  $k_{\max} \approx 0$  at onset and  $k_{\max} \rightarrow 0.68$  for larger values of the control parameter (Goldstein *et al.* , 1996).

#### 4.2.4 Marginal stability criterion and linear response

Most theoretical work to date has focused on the initial, linear, instability (but see (Goveas *et al.* , 1997)). This represents a major obstacle to quantitative comparison of theory and experiment, since the linear stage cannot be stabilized experimentally, nor can its growth rate be measured directly. In fact the linear stage appears to be completely masked by fluctuations of the membrane, with similar amplitude and wavelength as the instability. We are thus driven to try to use linear theory to describe nonlinear phenomena, and this is enabled by the existence of propagation of the instability outward from the point of tweezing, in both directions along the tube. It is well known that for a variety of nonlinear systems in which a stable, patterned state (in our case the pearls) invades an unstable, uniform one (the straight, tense tube) the selected velocity and wavelength can be found from the linear dispersion relation, using the “marginal stability criterion” (MSC) (Dee & Langer, 1983; Fineberg & Steinberg, 1987; van Saarloos, 1988). This works because the front at its leading edge has small amplitude, and obeys the linearized equation of motion. Using the MSC, numerical evidence has been given for the existence of a front, along with precise analytic predictions for the critical velocity and wavelength for tension  $\Sigma$  well above the threshold  $\Sigma_{\text{pearl}}$  (Goldstein *et al.* , 1996). One surprising feature of the analysis is that the selected wavelength is slightly different from the most unstable mode, leading to a deviation from the simplified model we sketched in §4.2.3; specifically, the velocity of propagation was found to scale as

$$\bar{v} \equiv V/V_{\kappa} = 0.082\Sigma/\Sigma_{\text{pearl}} , \quad (21)$$

where  $V_{\kappa} = \kappa/\eta R_0^2$ . Close to threshold one must solve the equations of (Goldstein *et al.* , 1996) numerically (T. R. Powers, unpublished calculation). A convenient interpolating formula approximately reproducing the solution is

$$\bar{v} \equiv V/V_{\kappa} = 0.082(\Sigma/\Sigma_{\text{pearl}} + 0.067) - 3713e^{-10.655\Sigma/\Sigma_{\text{pearl}}} . \quad (22)$$

Eqn. 22 is approximately valid for any  $\Sigma > \Sigma_{\text{pearl}}$ .

A different approach to analyze the propagation is to use linear response to calculate the response of

an unstable system to a localized, point-like perturbation (Gurin *et al.* , 1996). Due to dispersion the perturbation diffuses from the origin and is amplified by the unstable modes. This analysis predicts an identical scaling of velocity and introduces an experimentally measurable delay time which is the typical time for an appreciable amplitude to develop,

$$\Delta t = 33C\tau_\kappa \left( \frac{\Sigma_{\text{pearl}}}{\Sigma - \Sigma_{\text{pearl}}} \right). \quad (23)$$

where  $C$  is an experimental parameter measuring the amplification that is needed to bring the amplitude to an experimentally measurable value (Bar-Ziv *et al.* , 1997a).

### 4.3 Onset and propagation

We have recently reported a quantitative study of the onset of the instability and the propagation of unstable fronts (Bar-Ziv *et al.* , 1997a). The experiment was carried out on tubes made from SOPC. Tubes were produced by first depositing lipid on the bottom plate, closing the cell with a top plate and then filling the cell with water through small fill holes in the side, that were then sealed with epoxy. At the lipid structures were self assembling, a flow was induced by gentle rubbing along the top plate of the sample cell. Lipid globules detached as a result of the flow, and the tether they left behind usually evolved into a single-bilayer tube. We determined that it was a single bilayer by optical contrast and by tweezing, which easily separated tubes that were composed of many bilayers into the separate constituent bilayers. In this specific sample the tube diameters were relatively monodisperse, almost all of them in the  $1\mu\text{m}$  range. This is probably related to the uniformity of the drag force induced by a large scale flow. The concept of area loss by application of the tweezers was vividly exemplified on occasion by the formation of a small vesicle that was created inside the tubes upon prolonged tweezing. This vesicle typically had a radius comparable to that of the tube and was visible, differing from the fragments that we hypothesize to form an osmotic gas. Such cases were discarded in the analysis of propagation.

Experimentally, the onset of pearling occurred when the laser intensity exceeded a critical intensity  $I_{\text{pearl}}$  which we measured to be  $I_{\text{pearl}} = 10 \pm 4$  mW on tubes of radius  $R_0 = (0.6 \pm 0.1)\mu\text{m}$ . Using these values we obtain  $\Sigma_L = 4.5 \times 10^{-4}$  erg/cm<sup>2</sup> for the laser induced tension, eqn. (3), and  $\Sigma_{\text{pearl}} = 4.2 \times 10^{-4}$  erg/cm<sup>2</sup> for the critical tension of pearling, with errors of up to 40% in both. The rough agreement between the two estimates of the tension is a qualitative confirmation of our simple electrodynamic model for the

laser-membrane interaction.

In (Bar-Ziv *et al.* , 1997a) we used  $I_{\text{pearl}}$  measured for one tube to evaluate the control parameter<sup>3</sup>  $\epsilon \equiv (I - I_{\text{pearl}})/I_{\text{pearl}} = (\Sigma - \Sigma_{\text{pearl}})/\Sigma_{\text{pearl}}$  for tubes with 14 values of  $R_0$  in the range  $R_0 \approx 0.4 - 1.0 \mu\text{m}$ . For each value of  $\epsilon$  we measured the position of the front  $X(t) = V \cdot (t - \Delta t)$ , with  $t = 0$  the time the laser was turned on, and extracted the velocity of propagation  $V$ , the delay time  $\Delta t$ , and the selected wavenumber  $qR_0$ . We review the results for  $\Delta t$  and  $qR_0$  here for completeness (figures 9–10). In particular, Fig. 10 qualitatively supports the prediction of a continuous bifurcation with long wavelength at threshold (Bar-Ziv *et al.* , 1997a).

The determination of the front velocity as obtained from figures such as Fig. 4 relied on our ability to distinguish between a pearled state and a ‘straight’ fluctuating tube. The difficulty in determining the front position is visually apparent, and this is a ‘best case’ since the amplitude in Fig. 4 is actually nonlinear, and higher than the fronts that actually were used in the analysis. The arrowheads in this figure, as well those in Fig. 1 of Ref. (Bar-Ziv *et al.* , 1997a) approximate a straight line and demonstrate how a propagation speed was obtained. We found out that this was done best by eye and not by our computer algorithm. Since the trap was positioned in the center of the video field of view we typically obtained only 3 to 4 measurements of  $X$  vs.  $t$ . As a consequence, the determination of the front velocity is noisy and limited and in particular we are not sensitive to slowing down of the front at large distances from the trap.

For a more detailed comparison to the MSC, we now analyze the front velocity using the interpolation formula, eqn. (22). In particular, the behavior of pearling near threshold not only tests the MSC but also lets us say something about the possible folding energy  $\Sigma_c$  introduced in §2.4. Recall that  $\Sigma_c$  is a hypothetical packing cost per unit area for pulling material into the trap; if present, it reduces the effective laser-generated tension.

Rather than measuring  $I_{\text{pearl}}$  once and using it to calibrate all the other tubes, we will now simply fit the observed front velocity  $V(I, R_0)$  globally to eqn. (22) for a total of 30 trials. We take a generic form for the laser-generated tension,  $\Sigma = PI - \Sigma_c$ , where  $P$  is a fitting parameter. The discussion of §2.1 gave a rough expectation that  $P \approx 5 \cdot 10^{-5} \text{erg cm}^{-2}/\text{mW}$  (eqn. (3)), but  $\Sigma_c$  is unknown.

We wish to test several predictions from our theory: (a) MSC predicts *constant front velocity*. This

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<sup>3</sup>We also assumed that the packing energy  $\Sigma_c$  was negligible. We return to this point below.

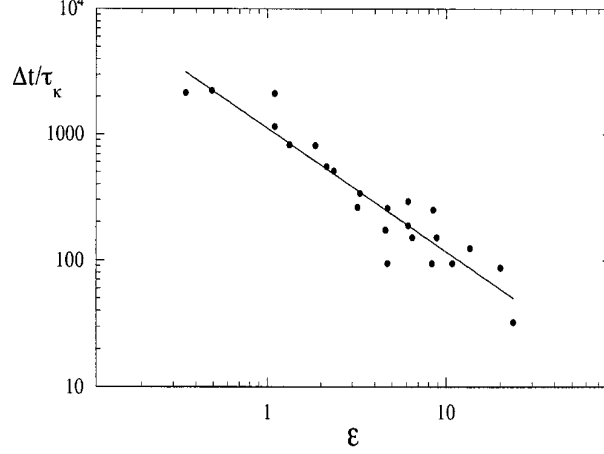


Figure 9: Delay time  $\Delta t$  scaled by  $\tau = R^3\eta/\kappa$  as a function of  $\epsilon$ . The best fit (solid line) yields  $\Delta t/\tau = (1000 \pm 200) \times \epsilon^{-1.0 \pm 0.15}$ . Taken from (Bar-Ziv *et al.*, 1997a).

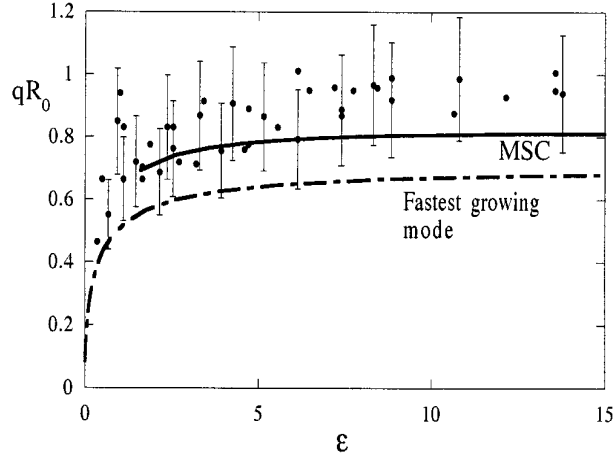


Figure 10: Selected wavenumber  $qR_0$  as a function of  $\epsilon$ . The theoretical prediction for fastest growing mode from the dispersion relation of GNPS (dashed curve) and their MSC wavenumber correction (solid curve) are also plotted. Taken from (Bar-Ziv *et al.*, 1997a).

is observed in every trial (data not shown). (b) MSC, combined with our dielectric model of laser action, also predicts a *linear relation between velocity and laser power* as long as the latter is well above threshold. (c) Moreover, eqn. (22) claims that *all the curves  $V(I)$  for various  $R_0$  collapse onto a single universal curve* when the dimensionless velocity  $\bar{v}$  is plotted against  $\Sigma/\Sigma_{\text{pearl}}$ . (d) Since  $V_\kappa$  and  $\Sigma_{\text{pearl}}$  both scale as  $\propto 1/R_0^2$ , the *slope of  $V(I)$  is a universal number*, independent of tubule radius. (e) As we just mentioned, we also have an estimate for the expected *value* of this slope.

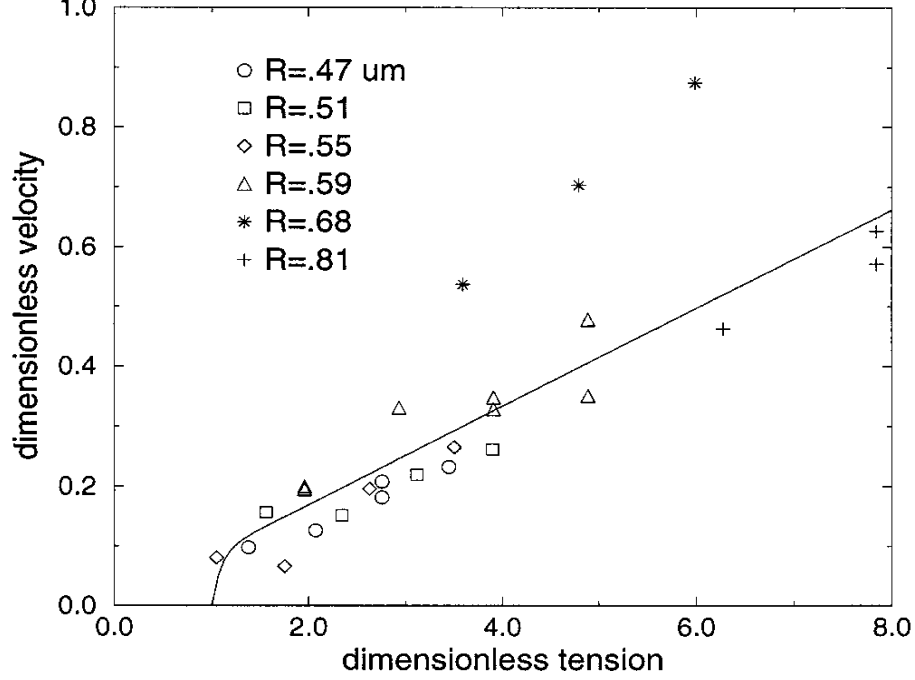


Figure 11: Dimensionless velocity  $V/V_\kappa$  of pearl propagation versus dimensionless tension  $\Sigma/\Sigma_{\text{pearl}}$  for various tubule radii  $R$ . The solid line is the theoretical prediction, eqn. (17), with  $\Sigma_c$  assumed equal to zero and one fitting parameter  $P$  (see text).

Figure 11 qualitatively confirms predictions (b) and (d). We carried out the fitting to  $P$  described above, again assuming  $\Sigma_c = 0$ . Evidently five of the six tubule radii shown collapse onto one universal curve. We have not explained the dissident tubule; note however that its  $R_0$  is not an extreme value. The data do not allow detailed assessment of (c) beyond the universality of the slope, but we note that pearling fronts do not propagate below threshold,  $\sigma < 1$ , consistent with (c). As for (d), the fit slope  $P = 2 \cdot 10^{-5} \text{ erg cm}^{-2}/\text{mW}$  is about half our rough estimate, eqn. (3).

We were not able to extract a definite number for  $\Sigma_c$ , due to the scatter in the data and random and systematic errors in measuring  $R_0$ . (Note that  $R_0$  drops out of the slope determination above.) We can, however, rule out the large value naïvely estimated in §2.4 above, in agreement with the anecdotal evidence mentioned there. Thus the exact process taking place inside the laser spot remains a mystery. One possibility is that the dielectric forces estimated in §2.1 pull material into the spot, whereupon enough energy is extracted from the beam to pulverize whatever material happens to be inside.

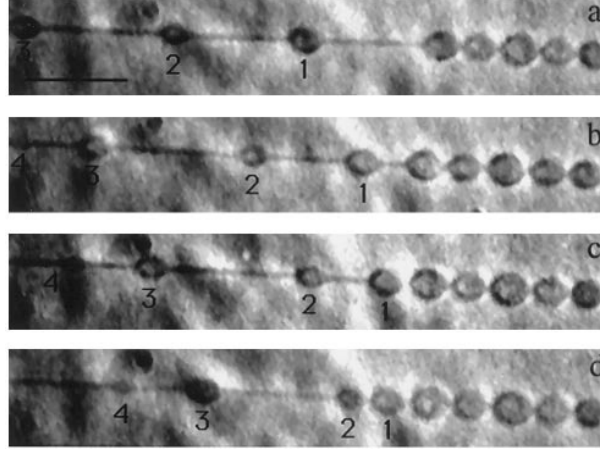


Figure 12: ‘Pearls on a string’ - nonlinear, late stages of an  $R_0 = 1 \mu\text{m}$  DMPC tube. Time between frames is 2 sec, starting about 20 sec after the laser was shut off. Bar is  $10 \mu\text{m}$ . Taken from (Bar-Ziv and Moses, 1994).

#### 4.4 Nonlinear stage

Longer or stronger application of the tweezers in the experiment leads to dynamic structures of isolated spheres that are interconnected by regions that have collapsed to very thin tubes ( $0.1 - 0.3 \mu\text{m}$  radius, depending on  $R_0$ ). While the initial instability propagates outwards, these spheres travel back, flowing along the tube towards the point of application of the tweezers where they aggregate (Figure 12). This motion typically continues for many seconds after the laser has been turned off. The velocity of a single pearl was constant to a very good approximation over the stretch we could measure ( $\approx 30 \mu\text{m}$ ), ranging between  $0.1 - 10 \mu\text{m}/\text{sec}$  for DMPC. However, with time (measured in terms of the number of pearls,  $N$ , reaching the cluster), the velocity decreased linearly, as shown in figure 13. This implies a similar reduction in the force acting on the pearls. It can be shown that a linear decrease with  $N$  indicates an exponential decrease with arrival time.

The spheres are taut as they move, all fluctuations damped by the surface tension. Eventually the pearls lose this tautness, but then they do not travel any more. The velocity of successive pearls as a function of time of arrival at the cluster is a slow relaxation decay consistent with an exponential time dependence with time constant of the order of minutes. We did not measure the velocities as a function of the laser intensity or tweezing time.

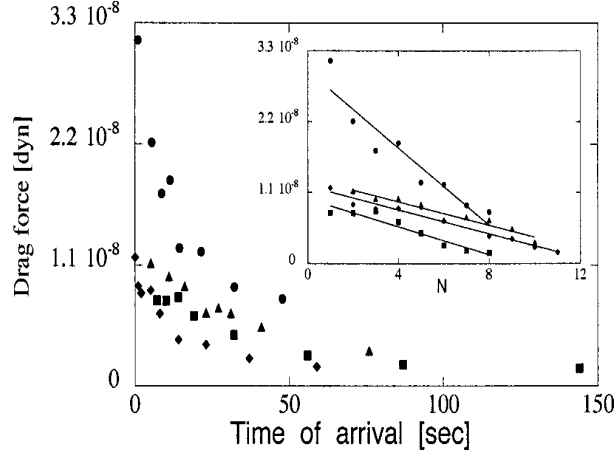


Figure 13: Measured velocities of successive pearls as they arrive at the cluster where the tweezers were applied. The velocities are normalized to give the Stokes drag force that they encounter,  $F = 6\pi\eta Rv$  with  $\eta$  the fluid viscosity,  $R$  the pearl radius and  $v$  the velocity. The forces are plotted as a function of both the time of arrival at the cluster and of the pearl ordinal (inset). Full circles designate data of DGDG while the rest corresponds to three different tubes of DMPC.

The motion of the pearls indicates a velocity scale  $V_p$  set by a gradient of pressure along the tube  $\vec{\nabla}P$ . For Poiseuille flow of water in the very thin tubes which the motion of the pearls causes,  $V_p = R_1^2 \vec{\nabla}P / 4\eta$  where  $R_1$  is the thin tube radius. Taking as a rough estimate  $\vec{\nabla}P \sim \frac{\Sigma}{R_1 L}$ , the Laplace pressure difference between pearl and tube over a typical distance  $L$  between adjacent pearls we obtain  $V_p \approx \frac{\Sigma R_1}{4\eta L}$ . For  $R_1 = 0.2\mu\text{m}$ ,  $L = 10\mu\text{m}$  and  $\Sigma = 10^{-3}\text{ erg/cm}^2$  gives  $V_p$  on the order of  $5\mu\text{m/sec}$ , in reasonable agreement with the measured velocities.

At this point, our experimental precision and control for the nonlinear regime are far below what we can reach in the linear stage. For this reason, we cannot compare our results quantitatively to the theoretical predictions of (Goveas *et al.*, 1997). They did, however, find qualitative agreement with some of our results. Their predicted values for the drift velocity of the pearls are of the same order of magnitude as the measured ones depicted in Figure 13.

We conclude the discussion on cylindrical instabilities by presenting a nonstandard case, in which the axial symmetry is not conserved. This instability was observed without any laser action, but is a rare occurrence. The lowest such mode (Gurin *et al.*, 1996) is an undulation of the cylinder. Figure 14 shows just such an excitation, probably produced by the sudden motion of one of the globules anchoring the tube. This would lead to excess area in the tube while conserving volume.

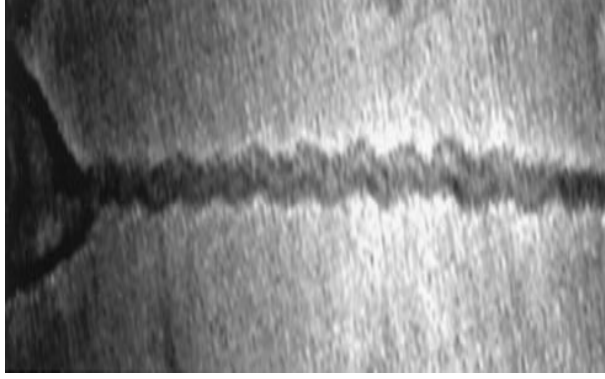


Figure 14: Undulation mode of a tube. This phenomenon was relatively rare, and in this case was produced in a manner unrelated to the laser tweezers. It was observed following large scale flow in the cell.

To the best of our knowledge, there has been no previous report of an undulation with a well defined wavelength in a fluid membrane tube. To see how this is possible we sketch a crude model. Excess area gives rise to a negative surface tension  $-\Sigma$ . Assuming as in the pearling instability that this tension is effectively uniform, we can write the energy gained per unit length by the tube when its axis wanders as  $\Sigma\delta A/L$ . If the axis wanders sinusoidally with wavelength  $2\pi R_0/q$  and amplitude  $a$ , then the time derivative  $\frac{d}{dt}\Sigma\delta A/L = \frac{\pi q^2 \Sigma}{2R_0} a \dot{a}$ . This energy gain in turn is spent on Stokes drag. For small  $q$ , we have roughly a force per unit length of  $4\pi\eta$  times the velocity. Equating the corresponding energy loss to the energy gain yields a growth rate of  $\dot{a}/a = q^2 \Sigma / 2\eta R_0$ , which vanishes as  $q \rightarrow 0$ . Thus perhaps surprisingly, the fastest-growing mode is at a *finite wavelength*, as observed in Fig. 14. Since the only length scale in the problem is  $R_0$  itself, it is not surprising that the chosen wavelength is comparable to  $R_0$ .

## 5 Laser induced expulsion in vesicles: an artificial machine on the micron scale

### 5.1 Summary

In this chapter we introduce the tweezers into structures with spherical topology. Two classes of transformations can be observed, each raising its own problems and exposing different physical mechanisms. *Spontaneous* expulsion of inner content will lead us to understand tension in a compact topology, and to a conjecture regarding the effect of detached membrane fragments as an osmotic gas. Expulsion under continuous action of the tweezers will lead us to invoke the suspension of lipid particles as a mechanism for



generating depletion forces, which can cause vesicles to slide and peel off each other. As a bonus, it led to the unexpected observation and measurement of the “buckling” instability of a spherical vesicle in an oscillatory process that is as yet unexplained.

Our preparation procedure of swelling lipids in water produces an ensemble of giant closed vesicles which often encapsulate many daughter vesicles. These complex structures can be simplified by a continual process of tweezing in which inner vesicles are expelled until one reaches intermediate forms of one mother-one daughter configurations. However, one often encounters such configurations without laser manipulation. Both spontaneously formed and manually manipulated configuration display identical expulsion processes.

For vesicles of closed spherical topology the action of laser tweezers induces expulsion of interior objects from a vesicle without observable damage to either the ‘parent’ or the ‘daughter’ object. This phenomenon is thus quite different from exocytosis or lysis, two processes in living cells. In exocytosis the interior vesicle fuses to the outer membrane wall, opens, and empties its contents outside the cell. In lysis, the outer wall bursts, releasing the inner object along with the rest of the cell content. Remarkably, in expulsion the interior object is completely unaffected and need not even be a vesicle; we have observed qualitatively the same phenomenon using glass beads. Equally remarkably, the exit pore which opens to release the inner object heals immediately without loss of the outer vesicle’s contents.

The conditions set up by laser tweezers can persist long after the laser is shut off and can lead to *spontaneous expulsion* of interior objects. We have reported this observation and proposed a mechanism based on the laser induced tension. *Spontaneous* expulsions have been observed only after the expelling vesicles have been transformed into *tense* spheres, as signified by the absence of visible fluctuations (Bar-Ziv *et al.* , 1995a; Moroz *et al.* , 1997).

Expulsion by lysis can readily be induced in bilayer vesicles by osmotic shock, using a sugar solution which produces a sudden pressure change (D. Kuchnir Fygenson, unpublished). This pressure difference is hard to estimate since the solute is released at a distant point from the vesicle and the concentration grows in a diffusive profile that cannot be meaningfully characterized. The surprising fact about the laser expulsion is that it is slow and nondestructive; furthermore this dramatic membrane reorganization happens for tensions estimated to be a thousand times smaller than what is normally required for rupture. Our main hypothesis in explaining spontaneous expulsion was that the laser trap pulls in lipid and ejects it in the form

of submicron objects, whose osmotic pressure then drives the process (Moroz *et al.* , 1997).

Expulsion can also be induced by *continuous* application of the tweezers: in this case one cannot decouple the mechanical state of the vesicle from the action of the laser as we did for spontaneous events. Surprisingly, in this case the outer vesicle is *not* tense, so osmotic pressure alone cannot be driving the process. Whereas spontaneous expulsion takes place typically within a fraction of a second, events in which the laser acts continuously can take up to a few minutes, during which there is a constant feedback between the state of the membranes and the constant influx of energy coming from the laser. We will describe in §5.2 below a single experiment in which an initially complex vesicular structure was simplified in two steps during which the laser was acting continuously. Thus far we have observed this exact process of events only once, so we cannot present a detailed model. Instead we will propose a qualitative explanation below.

In the first part of this section we review our results on spontaneous expulsion in greater detail than in previous reports. We then present quantitative image analysis of the more complex expulsions induced by active tweezing, and discuss possible explanations.

## 5.2 Spontaneous expulsion

Figure 15 shows 20 frames, 0.12 sec apart, of a typical giant spontaneous expulsion event. Several questions arise concerning the energetics and dynamics of such a process. What nucleates the pore, what drives the inner vesicle out, and what maintains the process? We will argue that once the exit pore opens the process is driven by the osmotic activity of a colloid of sub-micron lipid fragments created by the laser, and the corresponding Laplace surface tension. We will describe the stages leading to expulsion, elaborate on new measurements and review the model which has recently been proposed.

### 5.2.1 Laser induced rounding

Our experiments so far have indicated that spontaneous expulsion occurs only after the parent vesicle has been converted into a spherical shape. The typical effect of the tweezers on an initially flaccid vesicle is shown in Figure 16. Characteristic shape fluctuations of a floppy vesicle are shown in (a)-(c). The laser tweezers were then applied to the vesicle, (d)-(e), and during a period of about two minutes or so the fluctuations decreased until they were no longer apparent (f). At this point the vesicle had become spherical and quite taut, indicating that tension was present in the membrane.

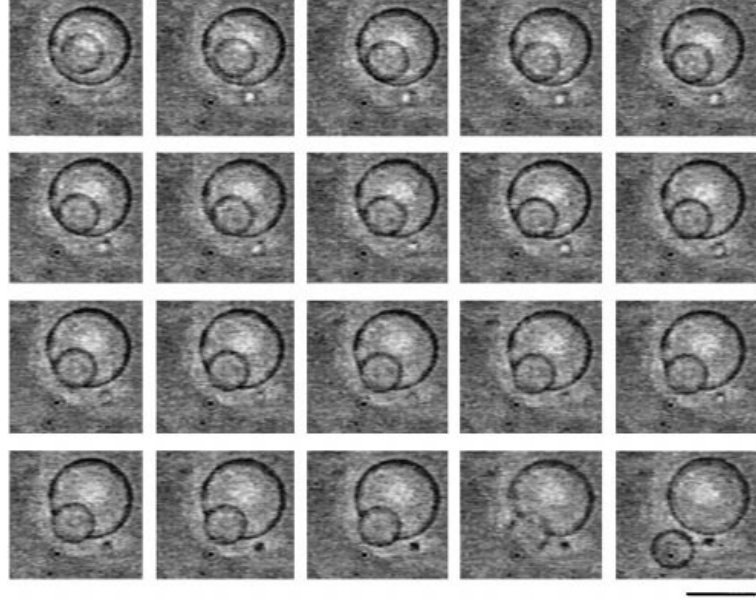


Figure 15: Spontaneous expulsion after laser tweezing. Concentric vesicles (SOPC) were produced spontaneously, often with multiple daughters. Both mother and daughter were initially in a floppy state, which had to be tweezed before arriving at the state shown at top left. Frames are 0.12 sec apart. Bar is 10  $\mu\text{m}$ . Time progresses from left to right and from top to bottom.

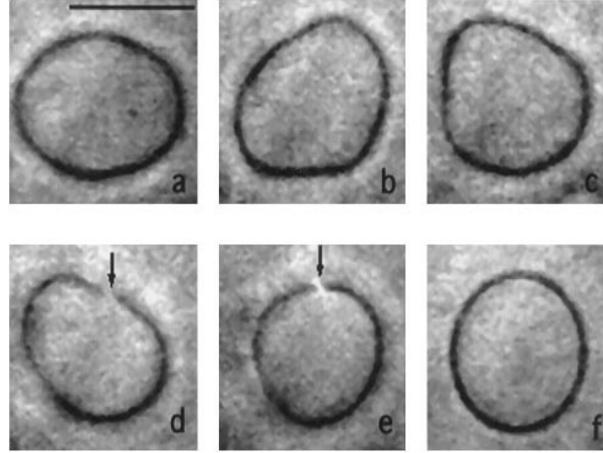


Figure 16: Laser induced rounding. (a-c) shows snapshots of the vesicle before tweezing, exhibiting typical thermal fluctuations. Tweezing (d-e, arrow marks point of tweezing) brought the vesicle to a spherical shape (f). Bar is 10  $\mu\text{m}$ . Taken from (Bar-Ziv *et al.*, 1995a)

To follow the increase of tension and approach to sphericity while tweezing, we extracted the contour of the vesicle during tweezing and measured the fluctuations in the normalized mean square of the radius of the vesicle (§3.3).

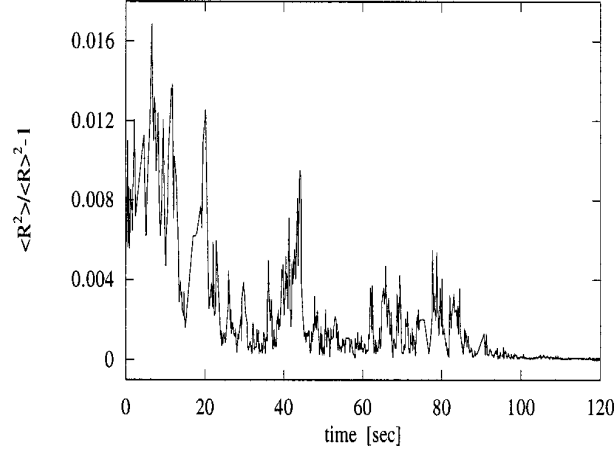


Figure 17: Time series of the normalized mean square of the radius of the vesicle while tweezing, reflecting the transformation of a floppy vesicle into tense sphere. Contours are 0.12 sec apart.

Figure 17 is the temporal evolution of the mean square radius for the same vesicle as in figure 16, showing strong fluctuations of the initially ellipsoidal floppy shape, superimposed on a gradual decrease of fluctuations as the shape approached the sphere, indicating an increase in surface tension. Vesicles pressurized in this way remain tense for hours. To obtain expulsion as in figure 15 we tweezed both the inner and outer vesicles till they were round and taut, and then turned off the laser to let the expulsion proceed on its own. Note that after 100 seconds the signal is flat, an indication of the reduced noise level obtained from the averaging involved in calculating  $\langle R \rangle$ .

### 5.2.2 Tension-induced adhesion and expulsion

Often the tense inner vesicle wandered for some seconds in Brownian motion before encountering the outer wall. Upon close enough approach, adhesive forces snapped the vesicles together rapidly, sometimes visibly deforming the outer vesicle towards the inner one before the two could draw together. Figure 18 shows snapshots of two vesicles, a few seconds after being tweezed. In this event we did not observe expulsion. After some wandering (a)-(b), the inner vesicle snapped to the outer one, deforming its shape (c), and then slowly relaxed back (d)-(f) while still adhering to each other.

Indeed, a measurement of the normalized mean square of the vesicle radius shows a rapid deformation of the outer vesicle followed by a slow relaxation of the deformed mode (Figures 18 and 19). Going back to Figure 15 we see that after a short waiting period the inner vesicle indeed began to emerge by first

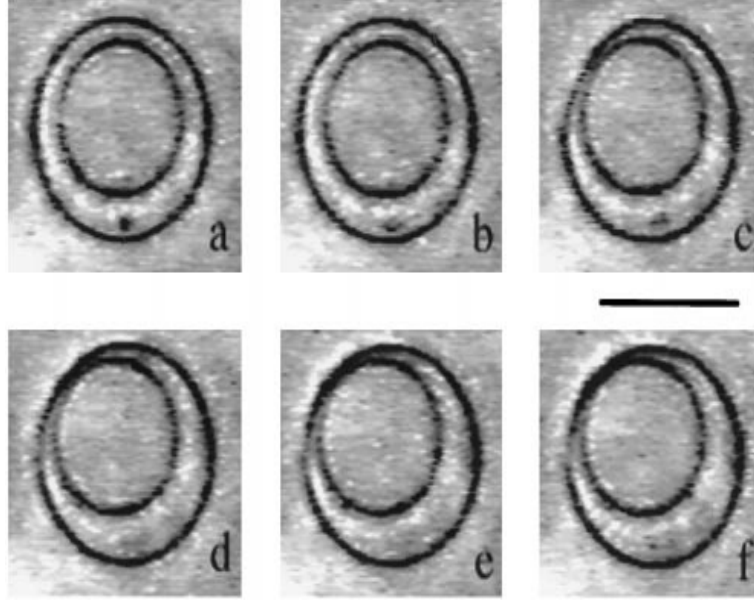


Figure 18: Tension-induced adhesion. Both vesicles have been laser tweezed. A strong deformation of the outer vesicle was observed (c) as it snapped to contact with the inner one. In (d)-(f) the deformation relaxed while the vesicles stayed stuck together. Frames are separated by 0.06 sec. Bar scale is 10  $\mu\text{m}$ .

snapping to adhesion. Tension-induced adhesion of vesicles is expected to occur very close to the spherical limit (Evans, 1985; Servuss & Helfrich, 1989; Lipowsky & Seifert, 1991; Seifert, 1995b; Helfrich, 1995).

In general, the point of initial tweezing was not related to the point of the subsequent exit. Though the daughter vesicle's volume was sometimes as large as 40% that of the parent, the parent always remained tense through the halfway point. In most events the daughter vesicle slowed considerably near the halfway point, and in some it stalled and retracted prior to this. Every vesicle which passed the halfway point completed its exit rapidly, in one or two video frames.

We analyzed another giant expulsion event, similar to Figure 15, and showed that the volume and area of the daughter remained roughly constant, from which we infer that at least one of its monolayer walls was intact throughout (Moroz *et al.*, 1997). Remarkably, the area of the *outer* vesicle also remained constant through the halfway point.<sup>4</sup>

Thus not only was the final surface area equal to the original, but at every intermediate step the outer

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<sup>4</sup>Actually we cannot measure the area or volume of a flaccid vesicle, since our method relies on knowing that the shape is spherical. What was observed is that the area is constant at least through the halfway point, since in all events the outer vesicle remained tense and spherical at least this far. For events remaining tense all the way to completion the area-conservation law held at all times.

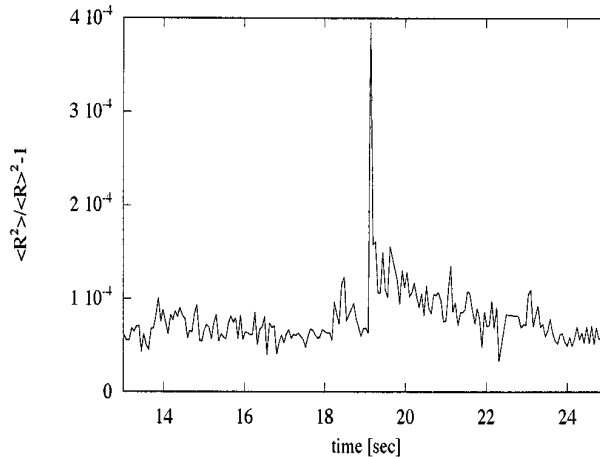


Figure 19: Tension-induced adhesion. Normalized mean square of the external vesicle’s radius during the snap to adhesion. The spike indicates the jump in the vesicle shape and is a rapid deformation, followed by a slow relaxation to the spherical shape. Data points are separated by 0.06 sec. The noise level of our digitizing algorithm is reduced in this averaging process. The noise can be estimated from the fluctuations up to  $t = 18$  sec, since the vesicle before buckling is perfectly spherical within our resolution. We see that a perfect sphere is identified to within under a percent of the radius, or  $5 \times 10^{-5}$  in these nondimensional units.

vesicle area (excluding the absent cap where the daughter is emerging) remained constant. Correspondingly, the volume  $\Delta V$  of the space between the vesicles was not constant but rather grew. In the final state the outer vesicle could end up flaccid (the case of a large daughter, (Moroz *et al.*, 1997)) or could remain tense (the case of smaller daughters, Fig. 15). We will present below a geometrical argument explaining when an expelling vesicle ends up flaccid and when it remains taut. The daughter was fully detached and could be readily pulled away by the usual tweezer manipulation; there was no tether connecting it to the outer vesicle.

Others have reported expulsion from vesicles tensed using the pipette method or osmotic shock (D. Zhelev, private communication; D. Kuchnir Fygenson, private communication). Their events seem to require far greater tensions than the ones reported here. Also, occasionally small vesicles with umbilical attachments can turn themselves inside out and thus appear to pass through a vesicle wall (E. Evans, private communication). We will return to this possibility below. Vesicle expulsion can also be induced by chemical means (Menger & Gabrielson, 1994). The phenomena we found are triggered solely by laser action. Finally, prompt laser-induced fusion of biological cell membranes has been reported by Schierenberg (Schierenberg, 1987); the mechanism appears to be quite different from the spontaneous rearrangement of pure lipid bilayers described here.

### 5.2.3 Model for spontaneous expulsion

For concreteness we will discuss the event shown in (Moroz *et al.*, 1997), where an outer vesicle of radius  $R = 4.5\mu\text{m}$  expels an inner one of radius  $r = 3.3\mu\text{m}$ . The direct observation of membrane tension  $\Sigma$  implies a corresponding hydrostatic pressure  $p = 2\Sigma/R$  inside the large vesicle, and yet water is clearly seen to be *entering*, not leaving, the intermembrane space. This is direct evidence for the osmotic flow mechanism described in §2.5: an excess of some solute in the interior maintains  $p$  while pulling more water in to dilute the solute (Oster & Peskin, 1992). With up to an atmosphere of osmotic pressure due to sugar on both sides of the outer vesicle, significant volume change *via* permeation through the bilayer is impossible. (Moreover the known permeation rate of  $70\mu\text{m}/\text{sec}$  for DMPC (Needham, 1995) is much too small to give the observed influx.) If however a gap of width  $w$  much larger than a sugar molecule opens, then small solutes like sugar become irrelevant. (We will return in a moment to the origin of the gap.) Indeed performing the experiment with a wide range of sugar concentrations had little effect on expulsion. As in §2.5, we propose that the relevant osmotically-active species is rather a suspension of membrane fragments.

For concreteness we will again illustrate our mechanism as in §2.5 using the smallest reasonable value for the size of these fragments,  $r_{\text{colloid}} = 5\text{ nm}$ . To estimate the volume fraction  $\phi$  we note that roughly 10% of the original  $250\mu\text{m}^2$  of surface disappeared permanently in the initial tweezing. Following §2.4 we suppose that it was converted to  $N$  spheres of volume  $\frac{4\pi}{3}r_{\text{colloid}}^3$ , about half of which are created outside the vesicle and escape. Since 10% of the original membrane occupied volume  $0.1 \cdot 250\mu\text{m}^2 \cdot 4\text{ nm}$ , we get  $N/2 \approx 10^5$  trapped spheres, or a volume fraction  $\phi \approx 2 \cdot 10^{-4}$ . The ideal gas formula (van 't Hoff's law) then gives an osmotic pressure  $\Delta\pi \approx k_{\text{B}}TN/2\Delta V = 17\text{ dyn}/\text{cm}^2$ .

The pressure  $\Delta\pi$ , and the corresponding Laplace surface tension  $\Sigma = \Delta\pi R/2 \approx 4 \cdot 10^{-3}\text{ dyn}/\text{cm}$ , must overcome a line tension  $\gamma_0$  for enlarging the exit pore. We can estimate  $\gamma_0$  directly by studying the rapid completion stage of expulsion. Here the exit pore snaps shut, propelling the inner vesicle a few microns in one or two video frames. From the pictures we estimate a speed  $v \approx 3 \cdot 10^{-3}\text{ cm}/\text{s}$ . Setting this equal to the Stokes drag on a sphere of radius  $r = 3.3\mu\text{m}$  gives the order-of-magnitude estimate for the line energy  $\gamma_0 \approx 1.4 \cdot 10^{-7}\text{ dyn}$ . Other events gave similar values.<sup>5</sup> As soon as the pore size exceeds  $\gamma_0/\Sigma \sim 0.4\mu\text{m}$ , the

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<sup>5</sup>Many authors have measured lipid bilayer line energies *via* electroporation and osmotic or hydraulic pressure in lipids different from ours, *e.g.* (Taupin *et al.*, 1975). While most obtain values larger than our direct estimate, all are within a factor of ten. It is possible that a contamination or conversion of lysolipid, perhaps due to the laser itself, reduces our line tension.

Laplace surface tension will be able to overcome the edge energy and the pore will grow.

Having identified a suitable driving force we now turn to the actual osmotic flow. We argued earlier that a macroscopic pore must open to defeat the volume-clamping effects of dissolved small solutes, which far outnumber our hypothetical membrane fragments. This pore must also be wide enough to admit water at the observed rate  $Q$ , as much as  $100\mu\text{m}^3/\text{s}$  (Moroz *et al.*, 1997), and yet small enough to trap the osmotically-active species. Since the tension  $\Sigma$  is far too small to create significant spontaneous pores, we instead focus attention on the rim of the parent vesicle. While the line energy  $\gamma_0$  tries to seal this rim tightly against the exiting daughter, thermal fluctuations constantly keep it open. We can estimate the average width  $w$  of the gap by adapting Helfrich’s “steric repulsion” argument (see (Lipowsky, 1995)). At the halfway point of expulsion, the rim of the exit pore is a tense fluctuating line, and so it feels an effective repulsive free energy of  $1.89 \cdot \frac{2\pi r(k_B T)^2}{\gamma_0 w^2}$  pushing it away from the daughter. The line tension however creates a two-dimensional “disjoining pressure,” or another contribution to the effective free energy of  $2\pi\gamma_0 w$ . Minimizing the total free energy gives an average gap width of  $w = 48\text{ nm}$ .

Thus the gap width  $w$  proves to be roughly of the right order of magnitude to allow free and rapid diffusion of small solutes like sugar, while still obstructing the colloidal particles and hence giving rise to the required osmotic flow.  $w$  is somewhat larger than  $2r_{\text{colloid}}$ , but there will still be an osmotic flow suppressed by  $2r_{\text{colloid}}/w$  (Finkelstein, 1987; Moroz *et al.*, 1997); recall also that 5 nm was just a lower bound on  $r_{\text{colloid}}$ .

We still need to estimate the *rate*  $Q$  of osmotic flow through a slit of width  $w$  and compare to the experiment. For a slit of length  $L$  in a very thin membrane, dimensional analysis yields an estimate for  $Q$  of  $pw^2L/\eta$ , where  $\eta = 0.78\text{ cP}$  is the viscosity of water at  $31^\circ\text{C}$  and we evaluated the geometric factor for concreteness at a pore radius of  $r/\sqrt{2}$ . Substituting the effective pressure  $p = \Delta\pi \cdot (2r_{\text{colloid}}/w)$  and  $L = 2\pi(r/\sqrt{2})$  gives  $Q \approx 14\mu\text{m}^3/\text{s}$ , comparable to but somewhat less than what is observed.

The conserved-area rule has an interesting geometrical consequence. Using it we can calculate the volume  $\Delta V_{1/2}$  between two spheres at the halfway point and compare it to the volume  $V_R = \frac{4\pi}{3}R^3$  of a tense full sphere with the original radius  $R$ . One finds that  $\Delta V_{1/2} < V_R$  for  $r/R$  greater than about  $2/3$ . Since the final stage of expulsion is rapid, there is no time for the required extra volume to enter, and hence the final state of the outer vesicle cannot be full. Indeed events like the one in (Moroz *et al.*, 1997) do end up flaccid. For smaller values of  $r/R$  the inequality is reversed: now there is no choice but to eject the excess water in a



jet, and we expect the final vesicle to be tense, again as observed (see Fig. 15). There is even direct evidence for the jet: small expelled daughter vesicles sometimes end up some distance away from the parent.

Finally we turn to the intriguing question of pore initiation. Bilayer vesicles can coexist in contact indefinitely in the absence of a specific fusogen such as  $\text{Ca}^{++}$  ions or polyethylene glycol, or a pulse of high electric field (Chang, 1987); membranes immobilized on mica plates must be forced together with pressures of thousands of atmospheres before they spontaneously fuse (Pashley & Israelachvili, 1981). Indeed in the absence of laser action vesicles retain included objects indefinitely with no spontaneous expulsion; vesicle expulsion without laser action (for example by pipette aspiration) requires tensions hundreds or thousands of times greater than those present here. What then creates the initial defect to nucleate expulsion?

We can rule out the formation of a defect at the laser spot since the exit pore has no preference to form there. Instead we speculate that the same submicron membrane fragments responsible for the osmotic activity also get trapped during the rapid jump to adhesion of the two large vesicles, and then provide an alternate pathway to expulsion, circumventing the costly small-pore stage. We get a hint of how this could happen when we notice that the primary barrier to membrane fusion is the hydration force between two intact membranes. But a 5 nm fragment is hardly a smooth, intact membrane. Its enormous curvature energy of  $8\pi\kappa \approx 1.5 \cdot 10^{-11}$  erg exceeds the line energy cost of cutting it into a flat disk, and so on average it will present large exposed hydrophobic regions. Such membrane fragments could in turn approach other membranes with less hydration repulsion than usual, and perhaps act as intermediaries catalyzing membrane fusion.

### 5.3 Expulsion by continuous tweezing: a single experiment

In this subsection we present an experiment where new kinds of expulsions were observed. Unlike spontaneous expulsion, these transformations required continuous driving by the laser. Although this process, in its exact sequence, was seen only once, quantitative analysis of its stages reveals a surprising new phenomenon.

The initial state shown in Fig. 20a consists of a tense bilayer ‘daughter’ vesicle trapped inside a tense *double* bilayer outer vesicle. This initial three vesicle configuration formed spontaneously in a floppy state and was pressurized by tweezing the inner and outer bilayers separately.

The experiment then consisted of two separate steps. In the first step (Fig. 21), the daughter vesicle

exited from the surrounding double vesicle. This induced the formation of a new kind of ‘fused hole’ in which the outer two ‘parent’ vesicles locally fused to form a curved fusion neck which prevented exposure of the hydrophobic part of the bilayer. The final stage of this expulsion was characterized by a slow closure of the fused hole, allowing free volume exchange between the interior and exterior of the parent vesicles (Fig. 21p). This was apparent from the gradual appearance of curvature fluctuations in the parent vesicles, in contrast with a simple pore, where rapid closure of the hole is driven by the high line tension.

In the second stage, after the daughter vesicle was fully detached and the fusion hole was closed, the two adhering vesicles were ‘peeled’ off from each other by continuous action of the laser (Fig. 22). This is a remarkable process, considering the fact that the two vesicles were of equal size! We analyzed both the inner vesicle and the outer engulfing vesicle and found that here, as in the first step, the outer vesicle did not remain tense during the entire process of peeling and strong fluctuations appeared close to the final stages of the expulsion. More surprisingly, the inner vesicle exhibited a periodic sequence of buckling deformations occurring every 5 sec, as the hole in the outer vesicle grew towards final expulsion (Fig. 22p). The origin of the periodic shape oscillations is not entirely understood, but their observation is unexpected and gives us an opportunity to measure the buckling deformation.

Figure 20 shows the intermediate steps of the experiment. First, a floppy membrane and a smaller encapsulated vesicle were brought into spherical shapes by the usual action of the laser. This is the starting point of the experiment (frame a). Then, the tweezers clamped together the outer and inner vesicles inducing a fusion neck between the two outer vesicles, which led to the final expulsion of the inner vesicle in a slow process described below (b). In the second step the tweezers were focused on the two bilayers (c) and by continuous action peeled them off (d). The reduction of actual area by tweezing in this step is clear, since twice the area of the initial vesicle was greater than the area of the two separated final vesicles.

### 5.3.1 First step: expulsion through a double bilayer

Expulsion through more than one bilayer produced a new kind of defect, a ‘fused hole’. While pores in a single bilayer are elastic due to the high line energy cost in exposing the hydrophobic core of the bilayer, the fused hole must be softer since it only involves the curvature energy. In fact, the energy of the fused pore is much lower than that of a simple pore, but the mean curvature is still greater than zero; as seen in Figure 21, the two principal radii of curvature are roughly  $4\mu\text{m}$  and  $1\mu\text{m}$ . Since it took the fused hole over

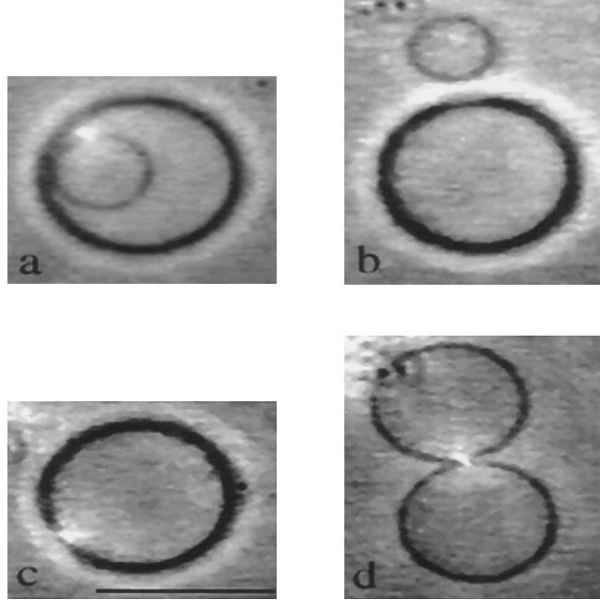


Figure 20: Starting points for the two steps. All three concentric vesicles were initially floppy (not shown). The tweezers were gently applied to pressurize all three and arrive at the state shown in (a). The top row depicts the first step starting with the daughter vesicle inside the parent vesicle (a). After the daughter was expelled (b,c), the two bilayers of the parent vesicle were subsequently separated (d). Bar is  $10\mu\text{m}$ .

a second to close, whereas an open pore closed within a video frame ( $1/25$  of a second), we infer that the effective line tension  $\gamma_{\text{eff}}$  of the fused hole is at least an order of magnitude smaller than the line tension of an open pore. This agrees with an order of magnitude estimate of  $\gamma_{\text{eff}} = \kappa/R_{lip} \sim 10^{-8}$  dyn, with  $R_{lip}$  the radius of the connection between the two concentric vesicles. This observation in turn implies that the surface tension needed to drive expulsion can be very low, since the energy barrier for pore formation is  $\Delta E \sim \gamma_{\text{eff}}^2/\Sigma$  (Taupin *et al.*, 1975). This equals about  $10k_{\text{B}}T$  for  $\Sigma = 10^{-4}$  erg/cm<sup>2</sup>, compared to about  $150k_{\text{B}}T$  for a true pore.

The expulsion of the daughter vesicle was assisted by the laser as it pivoted around the laser spot while exiting. As stated before, and seen in the figure, the hole did not close immediately after expulsion, but rather remained open for about two seconds before retracting, implying a free exchange of fluid. The presence of thermal fluctuations during expulsion shows that the tension is low.

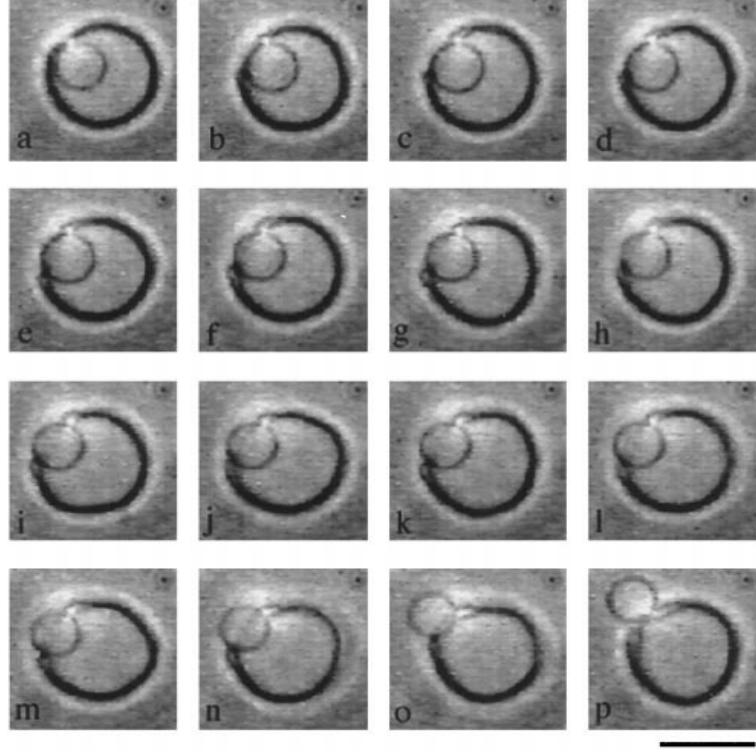


Figure 21: Time series of laser assisted expulsion through two bilayers. The two engulfing vesicles fused to make a pore, marked by its curved lips, which did not close immediately after exit of the inner vesicle. Fluctuations of the outer vesicles were observed during expulsion. The frames are 1.2 seconds apart. Bar is  $10\mu\text{m}$ .

### 5.3.2 Second step: Peeling and expulsion without tension

At this point we were left with a double-bilayer vesicle, which we subjected to further tweezing, with the ultimate effect of peeling the outer bilayer off, forming two separate unilamellar vesicles. This process was slow compared to spontaneous expulsion; it lasted 60 seconds, during which the laser acted continuously, clamping together the vesicles at a point. As separation proceeded the volume between the vesicles increased from nearly zero (initially, we cannot resolve the two bilayers) to a final value of about half the initial volume of the adhering vesicles. During this time the engulfing vesicle gradually slid over the inner spherical vesicle by the constant opening of a giant pore. To convey the dynamic nature of this remarkable process we show a time series of video frames separated by 3 seconds (Figure 22).

The laser point, marked by the bright spot on the left, is the place at which the pore initiated. As peeling progressed in time more area of the outer vesicle, which was initially bound to the inner spherical vesicle by

adhesion, was freed by the action of the laser. Especially close to expulsion we observed the appearance of thermal fluctuations of the outer vesicle, as seen towards the end of Figure 22.

The observation of fluctuations in Figs. 21 and 22 is a striking feature which rules out the possibility that surface tension, and hence Laplace pressure, drive the expulsion, in contrast to the situation in spontaneous expulsion, §5.2.3. This posed no problem in Step One, since the fused hole had a small line tension. In Step Two, however, we have a true bilayer pore. What then drives Step Two?

We find a possible explanation again using our colloidal-effects framework (see §2.5), remembering that the laser is in operation all the while. As we noted there, the volume fraction of membrane fragments in the very small volume between bilayers can be high, leading to an energy gain per unit area of up to  $0.01 \text{ erg/cm}^2$  when the pore creeps along the inner vesicle wall. Requiring that this gain exceed the cost  $\gamma_0 2\pi d(r_p)$  of increasing the pore size from  $r_p$  to  $r_p + dr_p$  and using the line energy  $\gamma_0 = 1.6 \times 10^{-7} \text{ dyn}$  estimated above, we get  $0.01 \text{ erg/cm}^2 \cdot d(\pi r_p^2) \geq \gamma_0 \cdot 2\pi r_p$ , which is indeed satisfied when  $r_p$  exceeds  $0.2 \mu\text{m}$ , a scale smaller than the laser spot size. Presumably the laser itself can disrupt the membranes to create an initial pore of this size.

Quantitative analysis of the peeling process is presented in Figures 23 and 24. The open contour of the outer vesicle was extracted between the starting point  $s = 0$ , close to the laser bright spot, and the end point  $s = s^*$ , which we chose manually as the point where the two membranes separated on the other side of the pore. We did not take into account the area of adhesion between the two membrane, which at all times conformed to the shape of the inner vesicle. We then computed the curvature and surface integrals of the contour. The main source of high frequency noise in these measurements is the digitization algorithm, rather than intrinsic fluctuations of the vesicle.

Looking at these two geometric quantities, we find a few striking features. The curvature integral defined in §3.3,  $\left\langle \left( \frac{d\Psi}{ds} \right)^2 \right\rangle$ , remained roughly constant for most of the process. Just before expulsion it increased rapidly, as most of the membrane was no longer bound and therefore free to fluctuate. In contrast, the surface integral  $\langle \Psi^2 \rangle$  did not change by much close to expulsion. Instead, it gradually increased from the beginning until approximately halfway through the peeling process. This measurement shows that at first, most of the energy pumped into the system by the laser was invested not only in increasing the pore size but also in unbinding the two membranes which were held by adhesive forces. Once unbound, the area measured

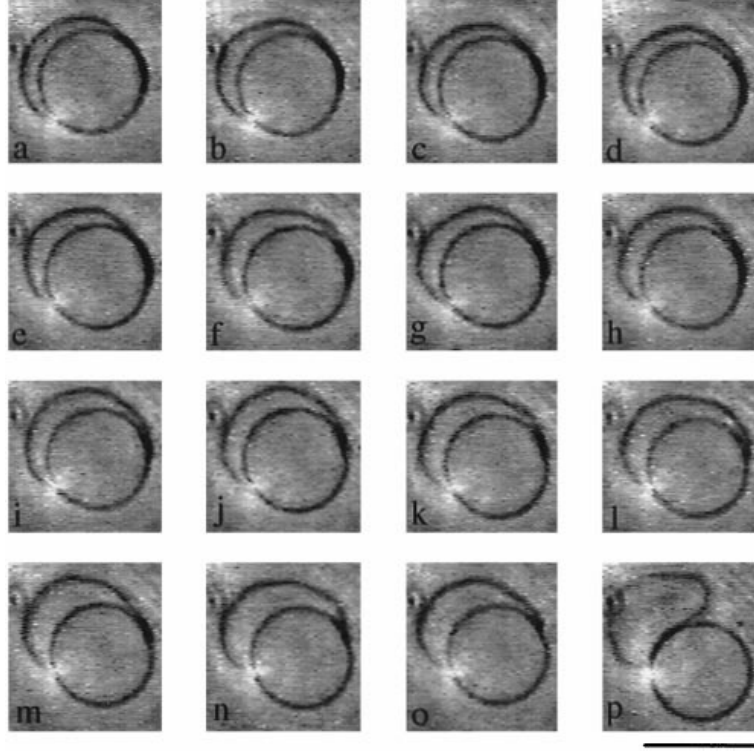


Figure 22: Second step - peeling a double bilayer vesicle. Video images taken every 3 sec. Bar is  $10\mu\text{m}$  and the bright spot is the laser trap. Time progresses from left to right and from top to bottom.

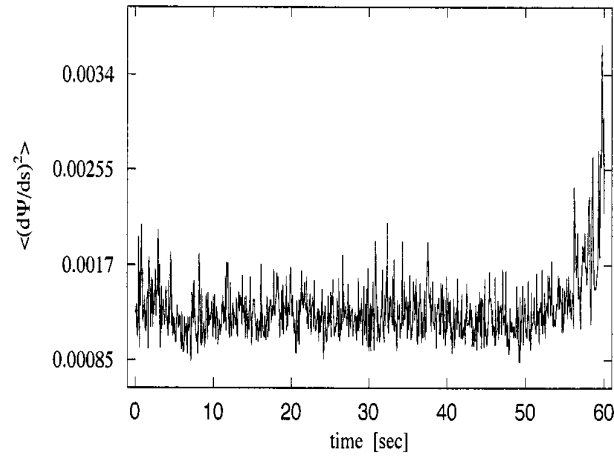


Figure 23: Time series of the curvature integral during Step Two (see text for detailed discussion). Time between measurements is 0.06 sec.

by our technique remains constant, and the vesicle is peeled by sliding the pore along the inner vesicle. The curvature fluctuations towards the end of the process indicate that not enough water is entering to keep the

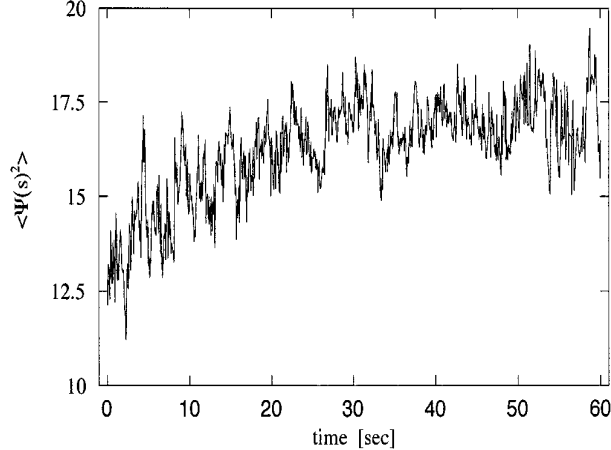


Figure 24: Time series of the surface integral during Step Two. Time between measurements is 0.06 sec.

inner membrane full as its edge creeps along the inner vesicle.

### 5.3.3 Buckling oscillations in the second step

Another, more subtle, feature observed in this peeling process is that the shape of the inner vesicle was *not* always spherical but underwent sudden ellipsoidal deformations, as demonstrated in Figure 25. As we show, these deformations displayed a periodicity of about 5 seconds and reflected recurrent buckling instabilities (Ou-Yang & Helfrich, 1988; Peterson, 1987). While the origin of the periodicity remains obscure, the opportunity to observe the dynamics of buckling cleanly and in a repetitive fashion is a rare one.

The buckling is most evident when we look at the ellipsoidal mode  $A_2$  in the expansion for the vesicle contour defined by eqn. (14) in §3.3. Figure 26 shows the time series of the magnitude of  $A_2$  over the entire process of peeling for one of the half contours of the inner vesicle.

The periodic peaks in the graph correspond to strong deformations when the vesicle buckled and hence the shape acquired a large  $A_2$  value. The periodicity of the oscillations is even more evident in the autocorrelation function of  $A_2$  as defined by,

$$\left\langle (A_2(0) - A_2(t))^2 \right\rangle, \quad (24)$$

which is shown in Figure 27. The oscillations are present also in the higher mode  $A_3$  but the signal is less pronounced and the signal to noise ratio is worse.

A reliable measurement of the line shape of the buckling instability is obtained by superposing all the

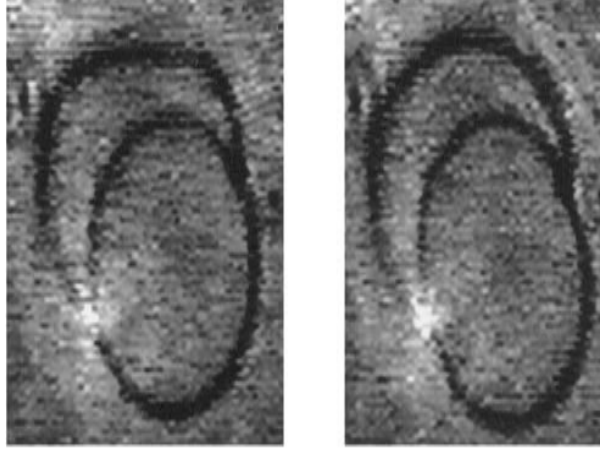


Figure 25: Two selected frames from the process of peeling, 0.12 second apart. In the first the vesicle is spherical, while in the second it has undergone a buckling instability. The outer membrane fluctuates, indicating a low surface tension.

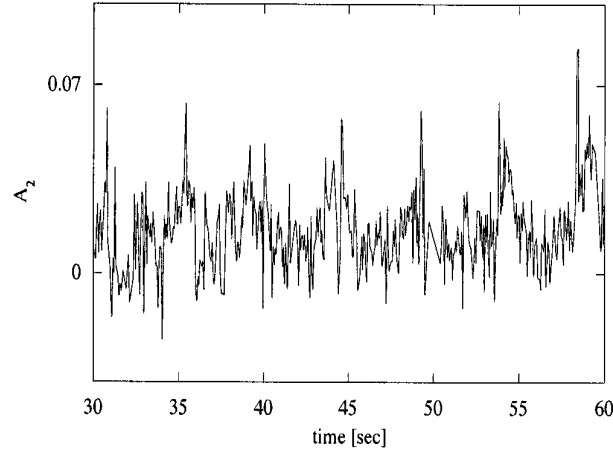


Figure 26: Time series of the  $A_2$  mode of the inner vesicle during the second half of the process. The peaks correspond to buckling deformations. Data points were sampled every 0.06 sec.

cycles of deformation, as shown in Figure 28. This clearly shows a time asymmetry in the shape of the peak, since the vesicle buckled in about 0.1 second and then relaxed back over about a second. This is because buckling of a spherical vesicle occurs as an *instability* above a threshold value of external pressure,  $P_c = -12\kappa/r_0^3$ , where  $r_0$  is the radius of the sphere (Ou-Yang & Helfrich, 1988; Peterson, 1987). This provides an estimate of the forces involved in buckling.

The origin of these buckling oscillations is not clear to us and awaits further investigation. We divide



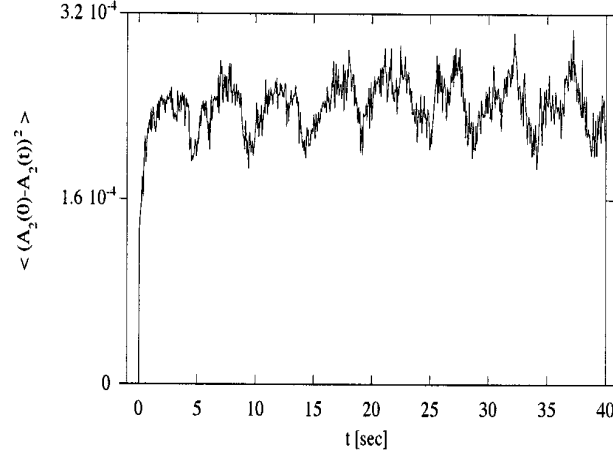


Figure 27: Time auto correlation function of the ellipsoidal mode,  $A_2$ , showing the 5 second periodicity.

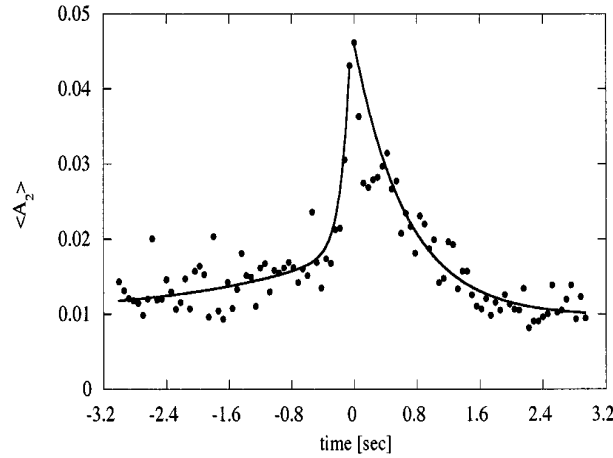


Figure 28: The average dynamic line shape of the buckling instability as obtained by a superposition of all the periodic deformations. Time  $t = 0$  is the frame at which  $A_2$  acquired a maximal value. The solid lines are each a sum of two exponential functions and guide the eye to indicate the different time scales of the excitation and the relaxation.

possible explanations into three general categories. (1) A local effect such as periodic escape of the membrane from the trap. (2) A global feedback mechanism of energy storage and release, where the vesicle buckles when the forces on it exceed a certain threshold. (3) Noise phenomena, such as mechanical vibrations.

The mechanical noise in our apparatus was monitored by looking at the flickering of the laser light reflected from the bottom plate of the cell. We selected a few square regions in the fringes produced by reflection of the optical trap from the glass bottom plate, typically five by five pixels, and measured the

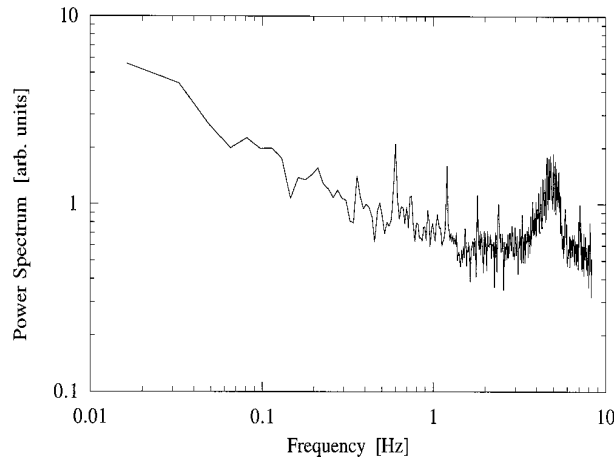


Figure 29: Power spectrum of mechanical noise obtained from the flickering of reflected laser tweezer light. A pronounced peak at 5 Hz is indicative of vibrations, with no indication of the 0.2 Hz buckling frequency.

average intensity reflected into that region. The scattering signal has a characteristic peak around 5 Hz, typical for mechanical vibrations, but no sign of a peak at the 0.2 Hz frequency of the shape deformations. We cannot, however, completely rule out other possible noise effects.

## 6 Excitations of nonspherical vesicles

### 6.1 Unbinding of planar membranes

Giant lamellar structures of bilayers are commonly observed after several hours of swelling, typically extending from the bottom to the top plates of the sample over hundreds of microns. These structures are mainly multi-lamellar and appear to be bound, or loosely bound within our optical resolution, due to kinetic constraints during formation, actual adhesion interactions or mechanical pressure. Such bound structures have been reported in the literature (Mutz & Helfrich, 1989).

When pinching loosely bound membranes together with laser tweezers we observed an unusual elastic response (Figure 30). The membranes locally unbound and separated to a large intermembrane distance. One could maintain such profiles in steady state with no observable tension effects away from the trap. This is because the lateral extensions of the membranes were practically infinite.

Menes and Safran introduced a theoretical model which incorporates bending elasticity, fluctuations and intermembrane interactions to calculate the membrane profiles subject to a local pinch, similar to a ‘sticker’

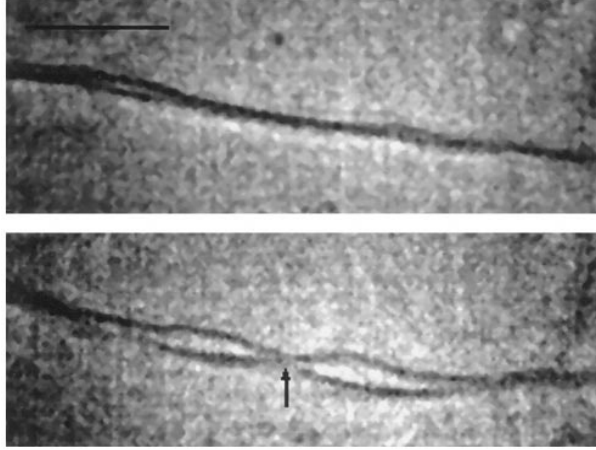


Figure 30: Local unbinding of pinched membranes. (Top) two bound membranes. (Bottom) Pinching by laser tweezers (arrow) cause unbinding of the pair. The bar is  $10\mu\text{m}$ . Taken from (Bar-Ziv *et al.*, 1995b), where additional details are presented.

which forces the membranes to be in close contact (Bar-Ziv *et al.*, 1995b). This model showed that the overall binding of two membranes with localized ‘stickers’ is strongly influenced by the elastic response and fluctuations of the embedding membrane, and is in general related to recent studies on membranes with inclusions (Dan *et al.*, 1994). The theoretical model produced unbinding membrane profiles, which were in good quantitative agreement with the experiment, using the pinch strength as a parameter. The theory went on to predict new scaling laws for the unbinding as a function of pinch strength and size. The experiment, however, could not check these predictions due to the narrow range over which the unbinding profiles were observed using video microscopy.

A natural extension of this work could be to study the interaction of two pinches as a model for interaction between adhesion sites embedded in membranes. While this approach has indeed been pursued theoretically (Menes & Safran, 1997), ‘optical pinches’, produced with laser tweezers, are not the best experimental model system to study the response of membranes to stickers. The major drawbacks of this approach are (i) Optical tweezers act continuously on the membranes, and possibly produce flow in the lipid itself and in the fluid around the pinch. (ii) The packing of lipids inside the pinch is below our resolution and we do not know the correct boundary conditions which are set at the interface of the pinch. (iii) The area lost from the trap into solution could be trapped between the membranes, producing osmotic effects such as in the expulsion experiments. Thus, to model local membrane stickers one should seek a more controlled system of mechanical

adhesion sites, for example by using coated beads.

## 6.2 Topological passages

Membrane “passages” are cylindrical necks which connect two membranes continuously, leaving no free edges. Shapes with passages in equilibrium have been a subject of intense experimental and theoretical research, in the context of the conformal invariance of the curvature energy (Ou-Yang, 1990; Mutz & Bensimon, 1991; Julicher *et al.*, 1993; Michalet *et al.*, 1994; Michalet, 1994; Charitat & Fourcade, 1997). In membranous cell organelles, such as the Golgi apparatus and the endoplasmic reticulum, passages are the basic topological building blocks which partition fluid volume by highly convoluted surfaces (Alberts *et al.*, 1989) (B. Fourcade, private communication). A passage between two concentric spherical vesicles creates a stomatocyte shape, while two such passages form a torus.

We show here, for the first time, how to go beyond passive observation to the *active creation* of such passages; instead of micromechanical means, we used laser tweezers.

Figure 31 shows a dynamic process in which a complex passage was formed. The initial configuration was a set of four floppy planar membrane contours obtained after a few hours of swelling, each corresponding to two bound bilayers. The laser, marked by the bright spot, was focused to a spot onto a pair of bilayers, pinching them together (a). The pinch locally separated the bilayers, creating a typical unbinding profile (b)-(c). As the overshoot grew it reached the upper pair and fused to form a passage (e)-(f). The laser was kept on, thereby widening the passage (g) and forming the remarkable structure of (h). At this point the laser was moved and then shut off. The structure was not stable and decayed in two consecutive steps (i)-(m).

While it is hard to obtain three dimensional information from these two dimensional figures, the unstable structure of (h) looks like a spherical vesicle inside a wide passage. However it is unlikely that this is indeed a sphere since it decayed spontaneously (i). Upon close inspection we see that the structure of (h) could be a new kind of topological excitation, which in the language of topology is a genus-2 defect. It can be envisioned as the fusion of two passages into one complex hole with two connections from one bilayer to the other. A view from the perpendicular side would have confirmed this conjecture.

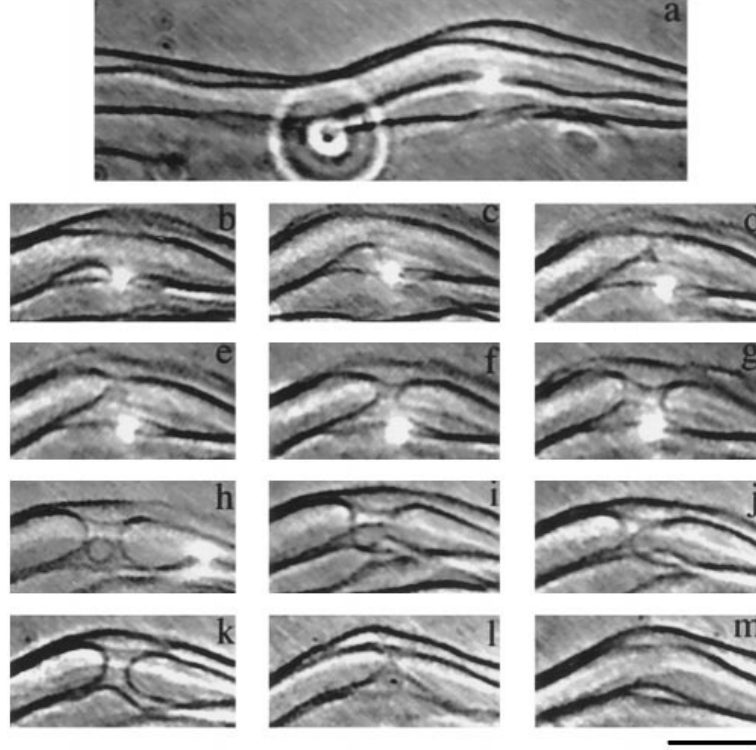


Figure 31: Excitation of a membrane passage using laser tweezers. Frame (a) shows a set of 4 contours each corresponding to 2 bilayers bound at an inter-bilayer distance below our separating resolution. Time of frames in seconds: (a) 0, (b) 13, (c) 21.5, (d) 22.1, (e) 22.6, (f) 22.3, (g) 23.8, (h) 26.9, (i) 28.4, (j) 29.1, (k) 30.6, (l) 45, (m) 71.8. Bar is  $10\mu\text{m}$ .

### 6.3 Shapes with high surface to volume ratio

Among the variety of vesicle shapes that form spontaneously in water are those which have a high surface to volume ratio such as the stomatocytes (Deuling & Helfrich, 1976). Theoretically, the shapes of vesicles are obtained as minima of the bending energy under the appropriate conservation laws and are parameterized by two physical variables, the dimensionless “reduced volume”  $v$ , and the area difference between the two monolayers comprising the bilayer,  $m_0$  (Miao *et al.*, 1994; Seifert, 1997). Transitions between equilibrium shapes have previously been induced by changes in temperature (Berndl *et al.*, 1990; Käs & Sackmann, 1991). Using laser tweezers, one can excite such vesicles by rapidly “quenching” the variables  $v$  and  $m_0$ . If the laser draws an equal amount of area from both monolayers then  $v$  must change, while removal of an unequal amount of lipid changes  $m_0$ . In the latter case even a small amount would suffice to excite large shape transitions. Tension is released by shape changes since there is ample area in the vesicle to compensate

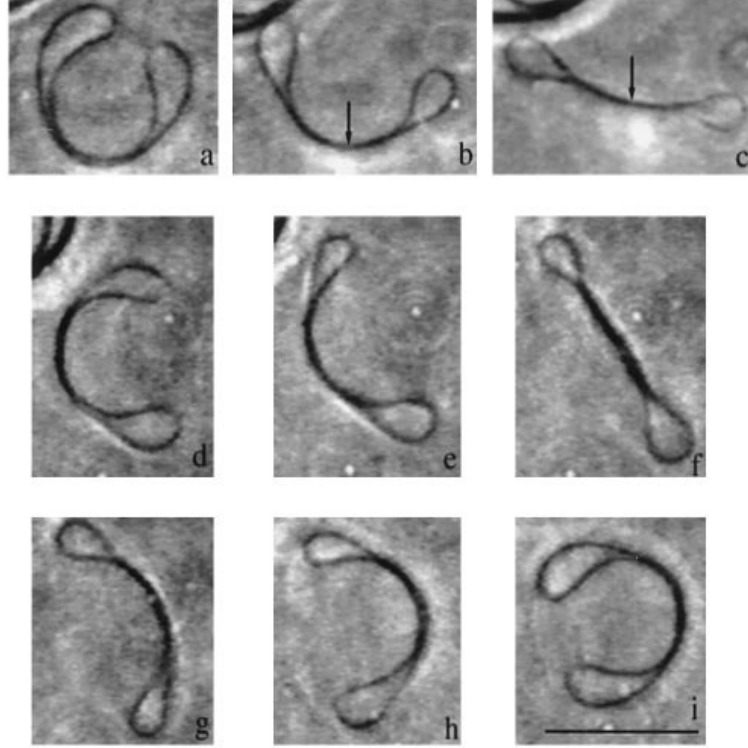


Figure 32: Damped oscillations of a stomatocyte. The stomatocyte of (a) was tweezed (b-c), bringing the vesicle to the symmetric shape. The point of trapping is marked by the arrow. The tweezers were then released. The vesicle first evolved to one symmetry state of the stomatocyte (d), but then went back through the symmetric state (f) to the opposite state (i). Axial symmetry is a good approximation for all the shapes in the figure.

for the loss and restore fluctuations.

As a result of the quench, the original stomatocyte shape is no longer the appropriate equilibrium configuration. The shape then has to dynamically relax, and we follow its trajectory in configuration space towards the equilibrium shape corresponding to the new values of the variables. Below we describe two particular examples of trajectories in configuration space during the relaxation of stomatocytes.

Figure 32 shows an initially floppy stomatocyte vesicle. Applying the tweezers on the vesicle caused it to transform into a rimmed pancake-like shape with restored up-down symmetry. After the laser was shut off the shape slowly oscillated between the two symmetry states of the stomatocyte configurations till it relaxed. Typically we observed 1-2 oscillations over a time scale of a few minutes.

The whole process of tweezing and relaxation was repeated twice for this vesicle, and on the third try

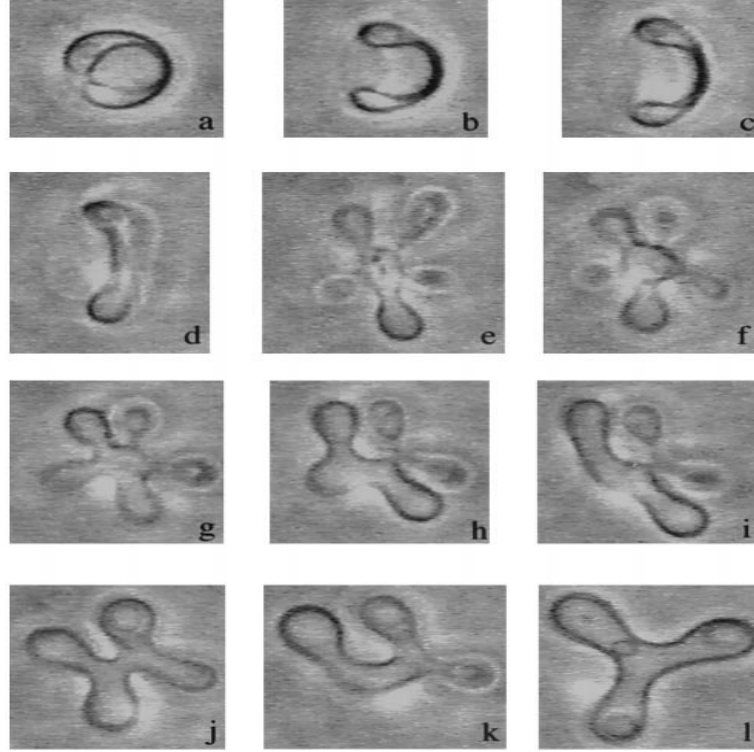


Figure 33: Starfish excitations. The original stomatocyte was tweezed in (a-c). The bifurcation occurred between (d) and (e). Time of frames in seconds: (a) 0, (b) 3, (c) 4, (d) 5, (e) 5, (f) 32, (g) 49, (h) 57, (i) 59, (j) 165, (k) 176, (l) 330. The laser was turned off in (c). The vesicle continued evolving beyond the three-fold starfish of (l) into a two-fold shape (not shown).

the shape diverged into a completely different trajectory with no apparent symmetry. We have found no thermodynamic argument for the existence of these damped oscillations, nor any dynamic mechanism that would account for them or for the long time scale.

In the second case, shown in Figure 33, the dynamic trajectory was completely different. Another stomatocyte vesicle was chosen and tweezed. As the vesicle approached the symmetric shape it underwent a striking bifurcation into a multi-finger starfish (Wintz *et al.*, 1996), breaking the axial symmetry. The laser was then shut off. We then observed a gradual coarsening of the shape, as the star-fish lost its arms one by one.

A possible explanation (T. Tlustý, private communication) for the appearance of the star-fish is the occurrence of a pearling instability in the rim of the stomatocyte. Assuming that the vesicle has no time to adjust its shape, tension may be built up for a short time. If at this time we regard the rim alone, it is

a toroidal vesicle that can undergo pearling. One can then obtain the number of arms in the starfish as a natural consequence of the area reduction. The volume and area of the cylindrical rim are:  $V = 2\pi^2 r^2 R$ ,  $S = 4\pi^2 r R$ , where  $r$  is the small radius of the torus and  $R$  its big one. Ignoring the central part, a final star-fish with  $z$  spherical arms and the same volume  $V$  has area  $\bar{S} = (144\pi^5)^{1/3} z^{1/3} (Rr^2)^{2/3}$ . To reduce area, we must comply with  $\bar{S}/S \leq 1$ , from which we obtain an upper limit on the number of arms  $z \leq \frac{4\pi}{9} \frac{R}{r}$ . Inserting  $\frac{R}{r} \approx 4$  from Figure 33 (c), we get  $z \lesssim 6$ , in agreement with the observed shapes.

## 7 Summary and conclusions

This paper combines previously unreported experimental observations with some new theoretical ideas. We have measured the optical force needed to bend a membrane, confirming the energy scale of the laser - membrane interaction. We then summarized the pearling instability and improved the analysis of the velocity scaling. We presented evidence of pearling induced by mechanical means (attaching to a bead or a micropipette and pulling on them, or by inducing a flow). A measurement of pearl migration in the nonlinear regime, and a first observation of a spontaneous transition to the first asymmetric mode (undulation at finite wavelength) of a tube were presented.

The discussion of expulsion in vesicles divides into a part dealing with spontaneous expulsion after tweezing, and a part dealing with expulsion under continuous tweezing. We have quantified the steps leading to spontaneous expulsion: a measurement of the gradual decrease of fluctuations while tweezing, and a measurement of the snap to adhesion as a precursor to expulsion. In the second part we analyzed in detail the consecutive expulsion of three concentric vesicles. This uncovered phenomena such as the fused hole formed between adhering bilayers, and “buckling oscillations” as one vesicle is peeled off another. The buckling instability was quantified and its full dynamic trace measured.

We reported the first observations of mechanical excitation of topological defects, in the form of fused necks between parallel bilayers. Excitation of stomatocytes leads to relaxation via novel oscillatory behavior and to a cascade of exotic starfish configurations.

The theoretical picture we presented includes a comprehensive treatment of the folding of a membrane into the electromagnetic trap formed by the tweezers. As lipids are packed into the submicron spot they get repackaged and expelled into the solution. There they can act as an osmotically active suspension that



produces and maintains a pressure as expulsion proceeds.

We have introduced the idea of colloidal creeping and have shown that it may explain some of the motion of one membrane along another, as the laser is applied.

A main hypotheses, the repackaging of lipid into sub resolution membrane fragments, has no direct proof but much circumstantial evidence. This calls for possible experimental verifications. The best candidate at the moment is localized dynamic light scattering (LDLS)(Bar-Ziv *et al.* , 1997b) - a technique specially developed to probe dynamics on the micron scale in a specific single region. Attempts at using fluorescence failed due to the high laser intensities.

We do not at present have a microscopic understanding of what really goes on inside the trap, but observe the resulting effect of tension outside. Still, we may have missed essential ingredients associated with chemical processes involved in the laser membrane interaction.

The use of the laser tweezers enables a new approach for dynamic excitations in lipid bilayers. Ideally, this brings us closer to understanding nonequilibrium phenomena in cells and in biological membranes. The laser applies a weak tension pulling in material and then detaching surface area. This is just enough to excite shape transformations without catastrophically disrupting the membrane.

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